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ATTEMPTS TO PRODUCE DECIDUOMATA IN THE PREGNANT RAT[1]

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A TRAUMATIC stimulus to the uterus of the pregnant rat from the 4th to the 7th day of pregnancy produces a local endometrial reaction known as deciduoma (Allen, 1931). Stimulation *after* the 7th day does not elicit this response (Allen 1931; Selye and McKeown, 1935). Selye and McKeown (1935) have claimed that this loss of sensitivity is due to a "specific" placental inhibition because after "cesarean section" they were able to obtain deciduomata. Atkinson and Leathem (1945) have suggested that, more specifically, the maternal placenta (the decidual tissue associated with the placenta) is the source of the "inhibitory" factor responsible for this loss of sensitivity. Since we have recently demonstrated that the presence of decidual tissue does not prevent the formation of deciduomata (Peckham and Greene, 1947, in press) the subject was reinvestigated.

METHODS AND RESULTS

Sensitivity of the pregnant rat's endometrium to the traumatic stimulus. Ten female albino rats with the left ovary removed were mated. The day on which sperm were observed in the vaginal smear was taken as the first day of unilateral pregnancy. The endometria of the non-pregnant uteri were stimulated by passing a suture transversely through the uterine wall at four points, leaving it loosely tied *in situ*.

Five animals were thus stimulated on the 5th day of pregnancy and another five on the 9th day of pregnancy. In each group the uteri were examined for deciduomata four days later.

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In seventeen other unilaterally pregnant rats the endometrium was stimulated on the 7th day of pregnancy, either by threading as above, or by amputation of the pregnant uterus. The uteri were examined for deciduomata on the 2nd or 3rd day following stimulation.[2] This procedure is described more fully below since these 17 animals were the ones used in the second part of this investigation (vide infra).

TABLE I. RELATION OF THE PERIOD OF PREGNANCY TO DECIDUOMA PRODUCTION

Day of pregnancy	No. of rats	No. of rats with decid.
5	5	5 (100%)
7	17	4 (23.5%)
9	5	0 (0%)

The results of these experiments are presented in Table I. The endometrium is uniformly sensitive to stimulation on the 5th day of pregnancy, partially sensitive on the 7th day, and completely insensitive on the 9th day.

Attempts to produce deciduomata following removal of the pregnant uterus. Since Selye and McKeown (1935) have claimed that "cesarean section" on the 7th day of pregnancy permits production of deciduomata on the 9th day but did *not* permit their production on the 7th day their experiment was repeated.

In 12 unilaterally pregnant rats the pregnant uterus was removed on the 7th day after copulation.[3] On the 9th day the animals were reoperated upon. Since deciduomata formed at the point of amputation of the pregnant uterus in two rats they were removed from the experiment and the deciduomata checked microscopically. In each of the remaining 10 animals the non-pregnant uterus was stimulated by threading as before. On the 12th day after copulation they were examined for deciduomata. Five additional unilaterally pregnant rats were treated in exactly the same manner except that the non-pregnant uterus was stimulated at the same time that the pregnant uterus was removed (on the 7th day after copulation). These animals were sacrificed on the 10th day. A section of vagina from each of the 15 animals was examined microscopically for estrus changes. All the non-pregnant uteri were checked for deciduomata microscopically.

The results of these experiments are presented in Table II. *No deciduomata resulted from stimulation of the non-pregnant uterus on the 9th day after copulation even though the pregnant uterus with its contained placentas and decidua was removed on the 7th day.*

[2] While deciduomata probably reach maximum size four days post-trauma they are grossly evident two days post-trauma.

[3] Pregnancy was verified by microscopic examination of one implantation site from each animal.

The vaginae of 2 of these 10 rats showed early estrus changes; the remainder showed the mucification typical of pregnancy or pseudopregnancy. Obviously in at least 8 of these rats the failure to form deciduomata cannot have been due to a recurrence of estrus.

Two of the 5 rats stimulated on the 7th day, at the same time the pregnant uterus was removed, developed one deciduoma apiece; one

TABLE II. ATTEMPTS TO PRODUCE DECIDUOMATA FOLLOWING REMOVAL OF THE PREGNANT UTERUS

Day* pregnant uterus removed	Day non-pregnant uterus stimulated	Day of autopsy	No. of rats	No. of rats with decid.	No. of rats with estrus changes
7	9	12	10†	0	2
7	7	10	5	2	0

* Refers to day after copulation.
† Two rats (not included in table) formed deciduomata at site of operative removal of pregnant uterus and were sacrificed on the 9th day.

developed at the point of the suture stimulus and one at the point of amputation of the pregnant uterus as was the case in the two animals previously mentioned (see footnote Table II).

DISCUSSION

Our data as to the sensitivity of the pregnant rats endometrium to the traumatic stimulus is in agreement with that of Allen (1931). However, unlike Selye et al., we were unable to obtain deciduomata after stimulation on the 9th day after copulation when the pregnant uterus had been removed two days earlier. Also unlike these investigators we *did* obtain deciduomata as a result of stimulation on the 7th day after copulation. As our experiment was as nearly a direct repetition as was possible these differences are difficult to explain.

Since removal of the pregnant uterus, and therefore the placentas and decidua, does not permit a response to the traumatic stimulus on the 9th day after copulation other factors must be operating which normally prevent production of deciduomata on the 9th day of pregnancy. It has been suggested (Rothchild and Meyer, 1942) and denied (Atkinson, 1945) that this loss of sensitivity may be due to either the presence of insufficient progesterone or to excess estrogen. Further investigation is needed to settle this problem.

SUMMARY AND CONCLUSIONS

Sensitivity of the unilaterally pregnant rat's endometrium to the traumatic stimulus was determined on the 5th, 7th and 9th days of pregnancy. Deciduomata were obtained in 100% of animals on the 5th day, 23.5% on the 7th day and in 0% on the 9th day.

Removal of the pregnant uterus in 10 rats on the 7th day after

copulation did not permit the deciduomal response to stimulation on the 9th day.

It is, therefore, unlikely that the placentas or decidual tissue are the factors responsible for the lack of sensitivity to stimulus after the 7th day of pregnancy in the rat.

REFERENCES

ALLEN, W. M.: *Anat. Rec.* 48: 65. 1931.
ATKINSON, W. B.: *Endocrinol.* 35: 193. 1944.
ATKINSON, W. B., AND J. H. LEATHEM: *Proc. Soc. Exper. Biol. and Med.* 58: 283. 1945.
PECKHAM, B. M., AND R. R. GREENE: in press, 1947.
SELYE, H., AND T. MCKEOWN. *Proc. Roy. Soc., London, Ser. B.* 119: 1. 1935.

PRODUCTION OF SECONDARY DECIDUOMATA IN THE CASTRATE AND LACTATING RAT[1]

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A TRAUMATIC stimulus applied to the uterus of the rat or mouse induces a local endometrial reaction known as a deciduoma, which reaches maximum size in about four days. It has been established that progestational hormone is essential for the production and maintenance of the deciduoma and that excessive amounts of estrogenic hormone interfere with production and maintenance. It has also been both claimed (Selye and McKeown, 1935; Lyon and Allen, 1943; Atkinson and Leathem, 1945) and denied (Rothchild and Meyer 1942) that the presence of established deciduomata inhibit the development of further deciduomata. The purpose of this study was to investigate these latter conflicting claims.

METHODS AND RESULTS

The production of secondary deciduomata in the castrate rat. Ten adult female albino rats were castrated during the estrus phase of their vaginal cycles. Daily treatment with 1.0 mg. subcutaneous progesterone[2] in 0.2 cc. peanut oil was started on the next day.

In each animal the right uterus was stimulated in four places on the fourth day of treatment by passing a suture transversely through the walls and lumen, tying loosely and leaving *in situ*. On the eighth day a laparotomy was done, the number of *primary* deciduomata was noted and each was measured.[3] The left uterus was then stimulated at four points in the same manner as the right uterus, and the laparotomy incision was closed. The progesterone injections were continued through the eleventh day. On the following day the animals were anesthetized and the *secondary* deciduomata were counted and measured.

The results of this experiment are presented in Table I. It can be seen that each of the ten rats responded to the first stimulation with

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[3] Deciduomata were counted and measured only at the point of standard stimulus (the suture). The deciduomata were measured at right angles to the metrial antimesometrial axis at the point of greatest diameter to the nearest 1/64 inch (0.4 mm). All measurements were made by the same observer.

the production of primary deciduoma and that each responded with the maximum possible number (four). Nine of the ten rats also responded to the second stimulation by the production of secondary deciduoma; six animals responded fully, two formed three deciduomata, one formed only one deciduoma and one formed none, yielding a total of 77.5 per cent of the maximum possible number. It is apparent that *the presence of primary deciduomata did not prevent the induction of secondary deciduomata.*

With respect to diameter, the secondary deciduomata averaged 0.61 mm. smaller than the primary deciduomata, a difference which is statistically significant.

This significant reduction in size of the secondary deciduomata cannot be attributed to a specific effect of the pre-existing primary

TABLE I. PRODUCTION OF SECONDARY DECIDUOMATA IN THE CASTRATE RAT

No. rats =10	Day of stimulus*	No. rats with decid.	Ave. per cent response	Mean decid. diam. (mm.)	Diff.† and its st. error	t-ratio	Probability
Primary Decid.	4	10	100	4.38	0.61 mm. and		
Secondary Decid.	8	9	77.5	3.77	0.181	3.375	0.01

* Refers to day of progesterone treatment.
† Statistical method used: paired comparisons. Snedecor, *Statistical Methods*, Iowa State College Press, Ames, Iowa, 1946.

deciduomata until the possible non-specific effect of the prolonged treatment period with its additional surgical trauma has been evaluated.

Accordingly, thirteen additional rats were castrated in estrus and treated with progesterone exactly as before. On the fourth day of progesterone treatment they were subjected to a mock operation consisting of a laparotomy but with no endometrial stimulation. On the eighth day they were again operated and *both* uteri were stimulated in four places, each with thread. On the twelfth day the resulting deciduomata were counted and measured.

In addition four other similarly prepared rats were used to induce primary deciduomata on the fourth day, exactly as in the original experiment, except that *both* uteri were stimulated in four places each. The resulting deciduomata were counted and measured on the eighth day as before. This provided a total of fourteen animals in which primary deciduomata had been induced without preceding surgical trauma to be used for comparison with the above thirteen animals which were mock-operated.

The results of this experiment are presented in Table II. It can be seen that each of the fourteen non-traumatized rats responded

with primary deciduomata; all maximally except one of the new animals added to the group, which responded with seven out of a possible eight deciduomata, giving a total percentage response of 99 per cent. All thirteen of the mock-operated animals likewise responded with deciduomata; eight animals formed eight deciduomata, three animals formed seven, and two formed six, yielding a total response of 93.3 per cent.

The mean diameter of the deciduomata in the mock-operated rats was 0.62 mm. less than that of the non-traumatized animals, a reduction which is statistically significant and which is almost identical with the reduction observed previously in the case of secondary deciduomata. These results, therefore, indicate that *the reduced size of secondary deciduomata is due to a non-specific effect* and not to a specific effect of the pre-existing primary deciduomata.

TABLE II. THE EFFECT OF OPERATIVE TRAUMA AND PROLONGED PRE-TRAUMA PERIOD

Group	No. rats	Day of stimulus	No. rats with decid.	Ave. per cent response	Mean decid. diam. (mm.)	Diff.* and its st. error	t-ratio	Probability
1	14	4	14	99.0	4.36	0.62 mm. and 0.184	3.408	0.01
2	13	8	13	93.3	3.74			

* Statistical method used: pooled variance, Snedecor, 1946.

The production of secondary deciduomata in lactating rats. Ten adult female albino rats were mated, and observed carefully for the day of delivery, which was taken as the first day of lactation. The number of pups per litter was immediately adjusted to six and maintained at this level throughout the experiment. On the fifth day of lactation the right uterus of each animal was threaded in four places as in the preceding experiments. On the ninth day the resulting primary deciduomata were counted and measured and the left uterus was in turn stimulated. On the thirteenth day the resulting secondary deciduomata were counted and measured.

The results of this experiment are presented in Table III. It can be seen that each of the ten rats responded with both primary deciduomata and secondary deciduomata; in each case a total of 37 out of a possible 40 deciduomata were formed, yielding a total percentage response of 92.5 per cent. Accordingly, in the lactating rat as well as in the castrate rat, *the presence of primary deciduomata did not prevent the development of secondary deciduomata.*

The mean diameter of the secondary deciduomata, however, was

again less than that of the primary by 0.68 mm. This difference was not found to be statistically significant in contrast to the results with castrate rats. This is due to the greater standard errors which probably indicate that physiological control of progesterone levels in lactation is less uniform than that which may be obtained by progesterone injection in castrate rats. The mean reduction in diameter of these

TABLE III. PRODUCTION OF SECONDARY DECIDUOMATA IN THE LACTATING RAT

No. rats =10	Day of stimulus	No. rats with decid.	Ave. per cent response	Mean decid. diam. (mm.)	Diff.* and its st. error	t-ratio	Probability
Primary Decid.	5	10	92.5	4.62	0.68 mm. and	1.842	<0.10
Secondary Decid.	9	10	92.5	3.94	0.369		>0.05

* Statistical method used: paired comparisons, Snedecor, 1946.

secondary deciduomata, although not significant, is the same as that produced by non-specific means in the castrate and these same non-specific factors were operating in the lactating rats.

DISCUSSION

Primary deciduomata do not inhibit secondary deciduomata in the castrate rat. This is in support of the statement of Rothchild and Meyer (1942) which, however, was unaccompanied by data. Why Atkinson and Leathem (1945) were unable to obtain secondary deciduomata in five castrate mice treated with progesterone is not clear. It may be that a species difference exists between the rat and mouse. It is also possible that the non-specific factors of prolonged treatment and additional surgical trauma have a greater influence on the reaction in the mouse. Instead of merely decreasing the size of the secondary deciduomata, as in the rat, the effect may be to prevent their formation.

Selye and McKeown (1935) reported that they were able to obtain secondary deciduomata in only six of eleven (54.5 per cent) "normally lactating" rats and that these deciduomata were not "well formed." Lyon and Allen (1943) stated that their experiences had been similar to those of Selye and McKeown. On the possibility that this poor response was due to a lack of estrogen, they treated their lactating rats with estrone. Twenty-six of thirty-seven rats (70.2 per cent) responded with secondary deciduomata. This response is not significantly different from that of Selye and McKeown, nor is it significantly different from our 100 per cent response (chi-square method). The difference between our results and those of Selye et al. (54.5 per cent) is only of border-line significance (probability between 0.02 and 0.05).

This difference may be due to variations in the experimental conditions. Apparently in neither of the experiments referred to above were the number of suckling young adjusted to or maintained at a constant level during the experiment.

SUMMARY AND CONCLUSIONS

In nine out of ten castrate progesterone-treated albino rats, and in ten out of ten lactating rats, secondary deciduomata were successfully produced in the left uterus four days after the induction of primary deciduomata in the right uterus.

The mean diameters of the secondary deciduomata were 14 per cent less than those of the primary deciduomata. This reduction in size was due to the non-specific effect of the additional treatment period and surgical trauma. It was *not* due to a specific inhibitory effect which previous investigators have attributed to the already existing primary deciduomata.

REFERENCES

ATKINSON, W. B., AND J. H. LEATHEM: *Proc. Soc. Exper. Biol. and Med.* 58: 283. 1945.
LYON, R. A., AND W. M. ALLEN: *Anat. Rec.* 86: 417. 1943.
ROTHCHILD, I., AND R. K. MEYER: *Physiol. Zool.* 15: 216. 1942.
SELYE, H., AND T. MCKEOWN: *Proc. Roy. Soc., London. Ser. B.* 119: 1. 1935.

THE QUANTITATIVE RECOVERY OF SYNTHETIC ESTROGENS FROM TISSUES OF BIRDS (GALLUS DOMESTICUS), THE RESPONSE OF THE BIRDS' TESTIS, COMB AND EPIDERMIS TO ESTROGEN AND OF HUMANS TO INGESTION OF TISSUES FROM TREATED BIRDS

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IN THE present communication observations on some of the events following ingestion of derivatives of stilbestrol by male fowl are presented, particularly the distribution of ingested estrogens in the tissues, the effect on maleness, the rate of excretion and the events that may occur after ingestion of such tissues by humans.

Jaap and Thayer (1944) and Thayer, Jaap and Penquite (1944) (1945) observed a ready suppression of maleness after estrogen treatment in four to eight weeks old chickens but only slight response in old cocks. Noble (1939) in rats as well as Lorenz (1945) and Munro and Kosin (1940) in chickens recorded suppression of gonads by estrogens. Jaap and co-workers (1944) (1945) noticed improvement in skin quality which they ascribed to increased feed intake and fat deposition in the skin. Bullough (1944) (1947) and Bullough (1946) showed in mice, birds and fishes, that a characteristic response to endogenous as well as exogenous estrogen is a noticeable increase in mitotic activity in epidermis and in most tissues throughout the body. In response to estrogen Gassner (1946) found metaplasia in thyroids of rats and squamous epithelium throughout the genital ducts was demonstrated by Harsh, Overholser and Wells (1939). However, after long term treatment with estrogen Hooker, Carroll and Pfeifer (1943) found shagginess and loss of hair in their rats and quite thin skin with dermis as well as epidermis being involved. Burrill and Greene (1942) showed that androgens were markedly effective when absorbed directly into the peripheral blood stream but low or of no activity when implanted into the spleen. Cantorow et al. (1942) found that following intravenous injection of estrogens in dogs substances possessing 90–95

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per cent of the activity of the original substance could be demonstrated for 48–72 hours in the bile. Lehninger and Scott (1947) showed that slices of prostate suspended in Ringer-phosphate glucose medium absorb all of an added estrogen probably on tissue lipids; however when slices were used instead of prostate all the estrogen was destroyed. Strand (1939) demonstrated a 7.2 per cent to 25.2 per cent recovery of various injected estrogens in rabbit urine.

MATERIAL AND METHODS

Barred Rock or Leghorn cockerels of various ages were, when not otherwise specified, fed for 12 days in separate cages on the standard diet outlined in a previous communication, Bird (1946). To all rations was added 1 per cent of peanut oil in which the specified dosage of estrogens was dissolved.

Stilbestrol compounds and extracts of tissues and excreta were assayed using ovariectomized rats according to the method described by Pugsley and Morrell (1943). Mazola oil (a brand of corn oil) was used as the diluent in preparation of the dosage levels. The dosages were administered subcutaneously or orally at 9 a.m. and 4 p.m. and the smears were read 24, 41, 48, 65, and 72 hours after the last administration. The same compounds as those fed to the cockerels were used as standards in each case and the amounts recovered are expressed in terms of the respective compounds. Tissues removed for assay of estrogenic activity were minced, cooked on the waterbath, extracted with ether and after removal of the ether the residues were taken up in Mazola oil. Oils were rendered from adipose tissues of cockerels on the waterbath and poured through eight layers of cheesecloth. Recovery of estrogens from excreta was determined on material which had been oven dried at 100°C. for 24 hours, ground, extracted with ether and the residue taken up in Mazola oil. In cases where insufficient materials were available to do complete assays an approximation of the estrogenic activity was obtained by administering graded dosages to groups of four or five rats and the amount of the compound present was calculated from the regression line determined on a relatively large group of rats.

In clinical tests the estrogen containing tissues were fed to volunteers. For these experiments women free from apparent endocrine dyscrasias or gynecological conditions were chosen. All subjects were from eight to fifteen years post menopause. Response to the treatment with estrogen containing material was followed by the vaginal smear method of Papanicolaou (1943). A control smear was secured to establish the senile character of the vaginal secretion prior to initiating the experimental feeding.

Samples from birds for histological and other examinations were secured immediately after killing and dressing. Size of comb was used as one of the criteria for maleness and determined as length+height. Length was measured from the anterior contour to the posterior point of the blade and height from the base on the skull to the highest point of the serrations. Weight of testes was used as the second criterion of maleness, the right testis being taken in every case and weighed to the nearest milligram. Skin samples for histological examination were taken from the proximal surface of the tibial musculature of the right leg.

RECOVERY OF ESTROGENS

In order to show the relative estrogenic activities of the different compounds for rats and the precision of the results, the slope of the response curve (b), variance of slope (Vb) and the fifty per cent response (RD_{50}) are shown in table 1. These results show that diethyl-

TABLE 1. THE SLOPES OF THE REGRESSION LINE (B) AND THE RD_{50} OF THE ESTROGENS

	Administration	No. of rats	No. of curves	b	Vb	RD_{50}	
						Gamma	S.E. ×1.96 gamma
Diethylstilbestrol	Subcut.	181	3	7.74	2.97	0.32	0.27– 0.36
Diethylstilbestrol	Oral	425	6	11.53	1.24	2.02	1.94– 2.10
Dimethyl ether of Diethylstilbestrol	Subcut.	372	5	5.60	0.44	13.40	12.20–14.70
Dimethyl ether of Diethylstilbestrol	Oral	212	3	8.64	1.23	5.70	5.36– 6.04
Dienestrol diacetate	Subcut.	224	3	5.08	0.33	2.11	1.93– 2.29
Dienestrol diacetate	Oral	245	4	7.65	0.84	6.73	6.15– 7.34
Dimethyl ether of dienestrol	Subcut.	132	2	7.25	1.98	27.30	24.95–29.81
Dimethyl ether of dienestrol	Oral	80	1	6.69	3.09	20.50	16.04–26.07
Dimethyl ether of hexestrol	Subcut.	205	4	6.44	1.44	21.40	18.78–24.37

stilbestrol has a considerably higher estrogenic activity in the rat than the other compounds. It is also noted that the activity of dimethyl ether of diethylstilbestrol and dimethyl ether of dienestrol is greater by oral than by subcutaneous administration. This is contrary to results usually found with most estrogens. The slopes of the regression lines for these compounds are within the limits found for estrone by Pugsley and Morrell (1943) using a similar dosing schedule.

The recovery of dimethyl ether of hexestrol in the muscle, skin, liver, gizzard and adipose tissue (abdominal fat) of cockerels fed 13.2 mg. per cc. of the oil supplement for seven days is shown in table 2.

It is seen that estrogenic activity was found in all tissues of the treated cockerels whereas tissues from an untreated cockerel and a laying hen possessed no such activity. In view of the high concentration of estrogen in liver, skin and adipose tissue and since no effort had been made to free the muscular tissues from depot fats it was concluded that the estrogenic activity had been taken up mainly in tissue lipids. It was therefore decided to use the oil from adipose tissue as a convenient index in further studies.

TABLE 2. APPROXIMATE AMOUNT OF ESTROGEN AS DIMETHYL ETHER OF HEXESTROL EXPRESSED AS GAMMA PER 100 GM. IN TISSUES OF COCKERELS AFTER FEEDING 13.2 MG. PER 100 GM. OF FEED FOR 7 DAYS

Tissue	Cockerel 1	Cockerel 2	Cockerel 3	Cockerel untreated 4	Laying hen untreated
Muscle	43	125	60	0	0
Skin	260	260	2460	0	0
Liver	250	425	770	0	0
Gizzard	260	190	400	0	0
Adipose	—	—	5000	0	0

The adipose tissue was pooled from twenty cockerels which had been fed 13.2 mg. of either dimethyl ether of diethylstibestrol, dimethyl ether of dienestrol or dienestrol diacetate, per cc. of the oil supplement for 12 days and an additional group was given a total dosage of 10 mg. diethylstilbestrol in oil solution as daily subcutaneous injections. The results of the assays for estrogens are shown in table 3. Those for excreta are expressed on a dry weight basis. It is seen that the adipose tissue of the cockerels fed dimethyl ether of diethylstilbestrol contain a relatively high concentration of estrogen.

TABLE 3. THE RECOVERY OF ESTROGENS IN ADIPOSE TISSUES AND FECES

Compound	Feeding period days	Amount ingested mg.	Adipose tissue		Feces	
			M mg. per 100 gm.	Range (Sm ×1.96) mg./100 gm.	M mg./100 gm. (dry wt. basis)	Range (Sm ×1.96) mg./100 gm.
Dimethyl ether of diethylstilbestrol	12	241.8	4.28	3.87–4.73	—	—
Dimethyl ether of diethylstilbestrol	12	162.5	3.17	2.89–3.48	—	—
Dimethyl ether of diethylstilbestrol	12	166.0	2.47	2.25–2.71	18.8	16.2–21.7
Dimethyl ether of dienestrol	12	160.0	1.50 (app.)		20.8	17.9–24.1
Dienestrol diacetate	12	156.7	0		1.23	0.98–1.54
Diethylstilbestrol	Subcut. adm.	10.0	0		0.12	0.10–0.15
Control (untreated)	—	—	0		0	

In the case of dimethyl ether of dienestrol it was possible to obtain only an approximation of the amount present while in the case of dienestrol diacetate and diethylstilbestrol it was impossible to obtain any quantitative estimation of the amount present. With doses of 4 cc. of the fat the appearance of nucleated epithelial cells in the vaginal smears from the rats bore evidence to some measure of stimulation but in no case was a full estrus smear obtained. Hence it is considered that the amount of estrogen in this tissue after administering diethylstilbestrol and dienestrol diacetate is relatively small. The amount of estrogen present in the excreta in the case of the dienestrol diacetate and diethylstilbestrol is considerably lower than in the case of dimethyl ether of diethylstilbestrol.

The estrogen found in the adipose tissue and the excreta showed the same estrogenic properties as the estrogen fed. In the case of the dimethyl ether of diethylstilbestrol and the dimethyl ether of dienestrol it was observed that the estrous effect was of longer duration than

with diethylstilbestrol and dienestrol diacetate. Attempts were made to identify the estrogen spectrophotometrically and chemically in the adipose tissue and excreta but due to the other non-saponifiable materials still present after fractionation these attempts were unsuccessful.

CLINICAL TESTS

In order to evaluate the estrogenic activity of the tissues of the experimental birds, as applied to humans, controlled quantities of the estrogen containing tissues were fed which would furnish approximately known dosages of the estrogens.

The material fed in three cases consisted of livers from birds fed dimethyl ether of diethylstilbestrol, samples from comparable birds having previously been assayed and found to contain approximately 0.25 mg. per liver. Each test patient received eight livers per day for a period of 6 days, yielding a daily dosage of approximately 2 mg. of estrogen per day or a total dosage of 12 mg.

A fourth patient received oil rendered from adipose tissues of birds that had been treated with dimethyl ether of diethylstilbestrol. The oil was fed in the form of broth and butter substitutes in a dosage of 43 gm. per day. This dosage according to assay, contained 2 mg. of active material and hence was comparable to the approximate dosage of estrogen administered in a total of eight livers. A fifth patient was fed 43 gm. of oil from birds having been treated with dienestrol diacetate. According to assay the daily dosage of estrogen was in this instance 0.2 mg. or approximately one-tenth that administered to the previous four cases.

The response to this feeding of estrogens as mirrored in the cytology of the vaginal canal is shown in table 4. It will be seen that there is a definite tendency for the menopausal picture to be replaced by one characteristic of estrogenic activity. This change is exemplified by the disappearance of the basal type of cell and the appearance of the more differentiated squamous variety with varying degrees of cornification. This transition occurs in conjunction with decrease in the number of pus cells present.

Changes such as these were demonstrable in the cases fed both liver and fat, the daily amounts of which were adjusted to contain approximately 2 mgms. of estrogen. No cytological change could be detected in case #5 which received a daily estrogen dosage of only 0.2 mgms. per day. The observation, both subjective and objective, that the amount of vaginal secretion materially increased in this instance may be of some significance. The progressive changes in cytology which occurred in case #4 are shown in the photomicrographs reproduced in Plate 1.

RESPONSE IN TESTIS AND COMB

Three groups of 20 White Leghorn males were, at 10 weeks of age, placed in cages and fed a ration consisting of commercial chick starter.

TABLE 4. CLINICAL TESTS

Patient	Age at treatment	Age at last menses	Tissue fed per day	Estrogen daily approx. dose	Cell type in smear	Control	Therapy			Day 9	Day 11	
							Day 2	Day 4	Day 6			
1. G	63	?	8 livers DED*	mgm. 2	Basal Pre cornified Cornified Pus	2+ 2+ rare 3+	? 2+? 2+? ?	1+ 1+ 2+ 0	rare 1+ 3+ 1+			
2. D	56	48	8 livers DED	2	Basal Pre cornified Cornified Pus		0 ± + ±	2+ 1+ ± 2+	Few Few			
3. L	56	?	8 livers DED	2	Basal Pre cornified Cornified Pus	3+ 2+ 1+ 2+	± 1+ 2+ 2+	0 1+ 3+ 2+				
4. R	59	50	43 gm. fat DED	2	Basal Pre cornified Cornified Pus	+ + rare +	2+ + rare 3+	2+ + 2+	2+ 2+ +	rare 3+	2+ rare	Vaginal secretion greatly increased by day 6
5. T	55	38	43 gm. fat DiA†	0.2	Basal Pre cornified Cornified Pus	2+ 1+ 1+ 2+	2+ 1+ 1+ 2+	1+ 1+ 1+ 2–3+	1+ 1+ 1+ 3+	1+ 1+ 1+ 3+	2+ 1+ 1+ 2+	Vaginal secretion greatly increased

* DED—Dimethyl ether of diethylstilbestrol.
† DiA—Dienestrol diacetate.

Two groups received respectively 3.3. and 10 mg. dienestrol diacetate dissolved in each cc. of the oil supplement. The birds were distributed to the three groups by rotation according to body weight. After 12 days treatment the birds were killed and in table 5 are recorded the means of observations made on the three groups.

TABLE 5. THE RESPONSE TO ESTROGEN ADMINISTRATION IN GROWTH AND COMB AND TESTIS SIZE

	Weight 12 wks. (gm.)	Average total dose of estrogen (gm.)	Growth (gm.)	CV* (%)	Comb size (m.m.)	C.V. (%)	Testis (mg.)	C.V. (%)
Control	1174	0	245	20.5	120(78.0 42.7)†	17.7	950	91.6
3.3 mg.	1166	31.4	233	20.2	92(59.2 33.6)	11.7	167	25.3
10.0 mg.	1184	97.8	240	18.4	85(58.9 28.6)	13.4	135	20.2

* Coefficient of variability.
† Length height.

In the control group comb size varied from 101 mm. to 138 mm. and testis weights from 156 mg. to 2836 mg. with a significant correlation between the development of these two tissues as indicated by the coefficient $r = +0.61$. However, no relationship existed between development of the soma and of the tissues that normally are stimulated by endogenous androgen. The response to endogenous androgen stimulation is enormously variable in testis and quite moderate in comb development. In the two estrogen treated groups especially testes but also combs became atrophied with a consequent reduction in variability. There was no statistical difference between these groups which indicates that 3.3 mg. of the hormone used is about the optimum dose for White Leghorns at the age of 10 to 12 weeks. Both groups differed significantly from their controls except in growth which apparently was unaffected by the given dosage of this estrogen.

The ready response of comb and testis to estrogens in young birds was however found to decrease markedly with increasing age and sexual maturity. A group of 9 yearling males was fed 20 mg. dimethyl ether of diethylstilbestrol daily (1 cc. = 10 mg. morning and night except Sundays) which is approximately six times the dosage fed the younger birds described above. After one week all males produced normal fertility upon insemination to 8 hens for each male. After 15 days treatment it was found impossible to obtain semen from five

Explanation of Figures on Plate 1

FIG. 1. Control.
FIG. 2. Effect after feeding 2×2 mg. dimethyl ether of diethylstilbestrol.
FIG. 3. Effect after feeding 4×2 mg. dimethyl ether of diethylstilbestrol.
FIG. 4. Effect after feeding 6×2 mg. dimethyl ether of diethylstilbestrol.
FIG. 5. Effect three days after feeding was discontinued.
FIG. 6. Effect seven days after feeding was discontinued.

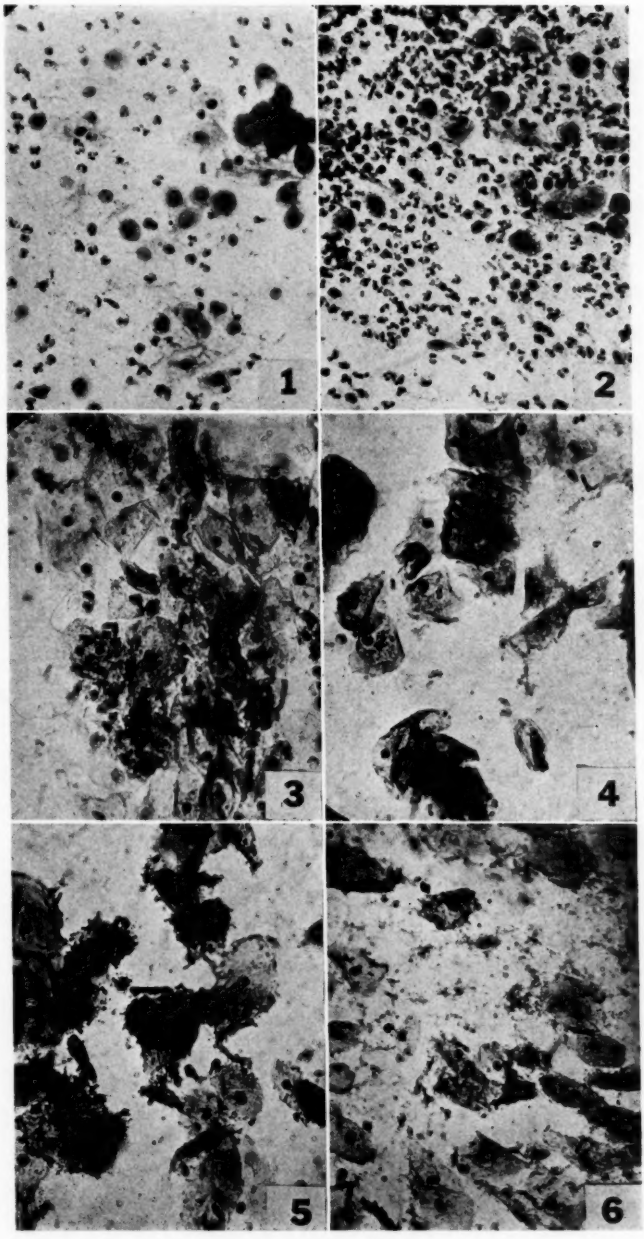

PLATE 1. Vaginal smears from menopausal woman Case #4. ×400.

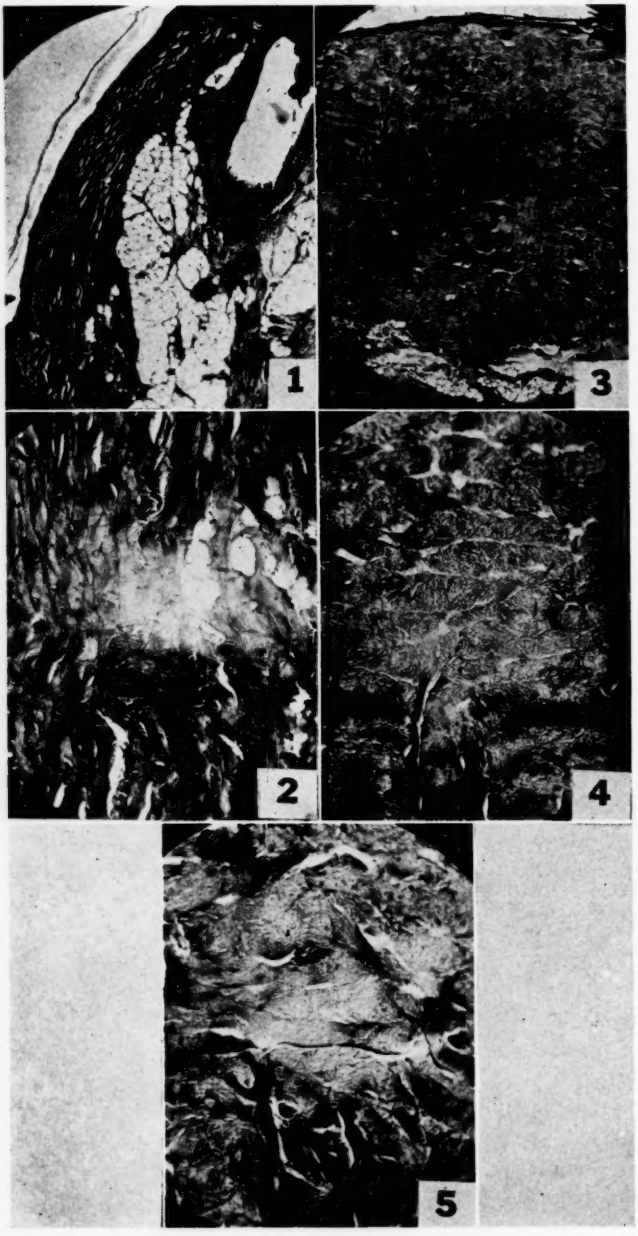

PLATE 2. Sections through epidermis of treated and untreated birds.

males and the remaining four gave low fertility. However, the testis of only two of the five which were killed weighed less than 4 gm. Histologically these atrophic testes were sterile, the lumen of all tubules being filled with younger generations of spermatogonia. In all testes above 4 gm. mature spermatozoa were found to be abundant. After three weeks only three males yielded semen but at six weeks, when the experiment was terminated, one male still retained large red head furnishings and produced copious amounts of semen. The testis of this bird weighed 14.9 gm. while the two others that were visibly affected by the estrogen treatment having pale and atrophied head furnishings and which only yielded a drop of semen after vigorous stimulation still had testes weighing 9.2 and 9.7 gm. respectively. Since the daily estrogen dose for all birds was exactly the same, but varied with length of treatment being from 7 to 18 times the average dose received by the younger birds it may be concluded that the variability in level of endogenous androgen must have been very high and the level itself must have been increased about 7 to 18 times with the increase in age from 12 to 52 weeks. Furthermore, it may be deduced from this experiment that 20 mg. per day is not the optimum dose for yearling males in the same way as 3.3 mg. is for 12 week old birds.

RESPONSE IN EPIDERMIS

One of our earliest observations, and since then the most constantly recurring one, has been concerned with the marked improvement in skin quality obtained consequent to estrogen treatment of male birds. The macroscopic characteristics of such high quality skin are its apparent smoothness and lucid whiteness. In order to study this character we placed three groups of six birds each, 28 weeks of age, on the standard treatment. The control group received the oil supplement only whereas the two experimental groups received respectively 13.2 mg. dimethyl ether of diethylstilbestrol and dienestrol diacetate dissolved in each cc. of oil. After 12 days the birds were killed and skin samples secured for histological examination. Photomicrographs of typical sections from the control and the dienestrol diacetate groups are shown in Plate 2. It is immediately apparent that the estrogen treatment has induced a marked hypertrophy in the strata making up the total epidermis. We selected in one section from

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Explanation of Figures on Plate 2

FIG. 1. Untreated control ×50.

FIG. 2. Untreated control ×200.

FIG. 3. Effect of treatment with 261.2 mg. dienestrol diacetate during 12 days. ×50. Compare total depth of epidermis in Figs. 1 and 3.

FIG. 4. Same section as Fig. 3. ×200. Compare small diameter of cells in Figs. 2 and 4.

FIG. 5. Same section as Fig. 3. ×200. Compare development of collagen fibres in Figs. 2 and 5.

each of the 18 birds three to four typical loci in which the short diameter of a cell was measured. The cells chosen were situated approximately equi-distance from the margin of the fat cells and the stratum corneum. Also the total depth of epidermis was measured. Mean values of the 20 to 23 measurements for each group are presented in table 6.

The smoothness and whiteness which are the characteristics of estrogen induced skin quality are therefore the macroscopic appreciations of thickening and consequent opacity of epidermal strata. In addition estrogen caused an increase in collagen material whereas the fat cells which underlie the epidermis were consistently diminished

TABLE 6. AVERAGE CELL DIAMETER AND EPIDERMAL DEPTH

	Average total dose of estrogen (mg.)	Single cell short diameter (micra)	Depth of total epidermis (micra)
Control		25	469
Dimethyl ether of diethylstilbestrol	241.4	60(50–70)	1209
Dienestrol diacetate	261.2	75(50–100)	1593

in size. The four birds, mentioned in the previous section, which did not respond in three to six weeks of estrogen treatment to the point of complete sterility of testis but which, in the case of three, presented capon-like heads all acquired remarkable skin quality. The conception therefore emerges that sensitivity to estrogen treatment in male fowl is highest in epidermis, lower in secondary sex characters and that the sex glands themselves possess a high and very variable resistance to estrogen therapy.

DISCUSSION

It has been shown (Table 1) that after oral administration the estrogenic potency of dienestrol diacetate is about equal to that of dimethyl ether of diethylstilbestrol and about six times higher when applied subcutaneously. In this respect these two differ perceptibly from other orally active compounds. In good agreement with these assays was the finding (Table 6) that skin changes were most pronounced after the dienestrol diacetate.

When the recovery of these two estrogens is calculated to total volume of excreta and estimated total weight of body fat and these values compared to the total weight of ingested estrogen it may be shown that the metabolic behavior of these two materials is remarkably different.

As regards the total recovery of dimethyl ethers of both diethylstilbestrol and dienestrol which in this regard behave in essentially similar fashion it seems probable that they are so poorly soluble that

most of these materials are excreted directly and that the absorbed fraction has the property of being quickly absorbed into the lymph system thereby escaping passage through the portal field with consequent destruction in the liver whereas 94 per cent of the diacetate is so destroyed. If this is the case it would seem that the necessary material for biological activity is but a minute fraction of the ingested amounts and would begin to compare with the values given by Albrieux (1941), Markee and Berg (1944) and Roberts and Szego

TABLE 7. APPROXIMATE RECOVERY OF INGESTED ESTROGENS FROM FAECES AND ADIPOSE TISSUE

	From faeces per cent	From fat per cent	Total per cent
Dimethyl ether of diethylstilbestrol	97.5	2.5	100.0
Dienestrol diacetate	5.58	0.13	5.71

(1946) for circulating estrogens. The material recovered from the excreta would then represent directly excreted material and in part a conjugated form of the metabolized fraction.

SUMMARY

Attempt to recover estrogenic materials from various tissues of treated birds showed that muscular tissues are very low in potency whereas tissues heavily infiltrated with lipids possess high potency. It appeared therefore that fractions of exogenous fat soluble estrogens are taken up chiefly by lipid containing tissues in preference to other tissues.

The recovery of five estrogens from the oil of adipose tissues from treated birds has been studied. After subcutaneous administration the estrogenic potency of these materials was, in descending order found to be diethylstilbestrol, dienestrol diacetate, dimethyl ether of diethylstilbestrol, dimethyl ether of hexestrol and dimethyl ether of dienestrol. After oral administration the dimethyl ether of diethylstilbestrol proved slightly more potent than the dienestrol diacetate but otherwise the order of potency was unchanged. Dimethyl ethers seem to have the property of being more potent orally than subcutaneously.

Assay of excreta showed that by far the greater portion of exogenous estrogens that can be accounted for are excreted direct. Clinical tests showed characteristic estrous changes of the cytology in vaginal smears after dosages of 6×2 mg. of dimethyl ether of diethylstilbestrol as livers or oils of adipose tissue from treated birds. When 6×0.2 mg. in oil from birds fed dienestrol diacetate was fed to a patient no change in cytology was observed but some effect was seen in the increased mucous discharge from the vagina.

Effective suppression of maleness in young cockerels 10 to 12 weeks old was obtained on a total dosage of 13×2.613 mg. estrogen

per bird. Testes as well as combs were affected. A threefold dosage of 13×8.145 did not have any greater effect and it is concluded that 3.3 mg. estrogen per 100 gm. of feed for 12 days is about optimum at this age of the birds. In untreated controls there was a highly significant correlation between size of the comb and development of testis. In 52 weeks old birds 20 mg. estrogen per day was not sufficient to suppress maleness completely but in spite of this partial failure, hypertrophy of epidermis was remarkable.

Secondary sex characters suffered regression more readily than the primary sex glands, spermatogenesis remaining quite vigorous even after the combs had acquired capon-like characteristics. Estrogen causes a marked hypertrophy of epidermal strata causing the surface of the carcasses to acquire a white and smooth appearance. The hypertrophic response of epidermis constitutes a more sensitive measure of estrogenic activity in male chickens than may be obtained from any other tissue so far studied.

ACKNOWLEDGMENTS

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REFERENCES

ALBRIEUX, A. S.: *Proc. Soc. Exp. Biol. and Med.* 47: 380. 1941.
BIRD, S.: *Endocrinology* 39: 149. 1946.
BULLOUGH, HELENA F.: *J. of Endocrinology* 3: 280. 1944.
BULLOUGH, HELENA F.: *Nature* 159: 101. 1947.
BULLOUGH, W. S.: *Phil. Trans. Royal Soc. Lond.* 231: 453. 1946.
BURRELL, M. W., AND R. R. GRENE: *Endocrinology* 31: 73. 1942.
CANTOROW, A., A. E. ROKOFF, K. E. PASCHKIS, L. P. HANSEN AND A. A. WALKING: *Endocrinology* 31: 515. 1942.
GASSNER, F. X.: *Endocrinology* 39: 61. 1946.
HARSH, R., M. D. OVERHOLSER AND L. J. WILLIS: *J. Endocrinol.* 1: 261. 1939.
HOCKER, C. W., AND CARROLL A. PFEIFFER: *Endocrinology* 32: 69. 1943.
JAAP, R. G., AND R. H. THAYER: *Poult. Sci.* 23: 249. 1944.
LEHNINGER, A. L., AND W. WALLACE SCOTT: *Endocrinology* 40: 9. 1947.
LORENZ, F. W.: *Poult. Sci.* 24: 128. 1945.
MARKEE, J. E., AND BRUCE BERG: *Stamford Med. Bull.* 2: 55. 1944.
MUNRO, S. S., AND I. L. KOSIN: *Endocrinology* 27: 687. 1940.
NOBLE, R. L.: *J. Endocrinol.* 1: 128. 1939.
PAPANICOLAOU, G. N., AND H. F. TRAUT: Diagnosis of uterine cancer by the vaginal smear. P. 3. Oxford University Press. 1943.
PUGSLEY, L. I., AND C. A. MORRELL: *Endocrinology* 33: 48. 1943.
ROBERTS, S., AND CLARA M. SZEGO: *Endocrinology* 39: 183. 1946.
STRAND, S. W.: *J. Endocrinol.* 1: 201. 1939.
THAYER, R. H., R. G. JAAP AND R. PENQUITE: *Poult. Sci.* 23: 555. 1944.
THAYER, R. H., R. G. JAAP AND R. PENQUITE: *Poult. Sci.* 24: 483. 1945.

THE RELATION OF THYROXINE TO TOTAL IODINE IN THE THYROID GLAND*

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THE WORK of many investigators has made it clear that 90 per cent or more of the iodine in the normal thyroid gland is present in organic combination. This organic iodine, according to Harington (1933), can be completely accounted for by thyroxine and diiodotyrosine. These two compounds do not normally exist free in the gland but can be isolated after suitable hydrolysis of the thyroid protein.

Various values have heretofore been reported for the proportions of thyroxine and diiodotyrosine present in thyroid tissue. Thus, by an acid separation, Harington and Randall (1929) obtained values of 40–50 for the ratio of thyroxine to total iodine. Similar values were found by others who employed their method (Rotter and Soos, 1933; Dey, 1945). Subsequently, Leland and Foster (1932), who used butyl alcohol for the extraction of thyroxine from an alkaline hydrolysate of the thyroid, reported that only 25 per cent of the iodine of the gland is present as thyroxine. Blau (1933) by modifying the method of Leland and Foster increased the thyroxine recoveries to about 30 per cent of the total iodine.

A recent adaptation of Foster's method to small amounts of thyroid tissue (Taurog and Chaikoff, 1946a and b) made possible a study of the influence of dietary iodine upon the thyroxine content of the thyroid gland of the rat. In rats maintained on iodine intakes ranging from 2 to 440 micrograms per day, the total iodine content of the gland rose from 21 to 131 mg. per cent, but the percentage found as thyroxine remained constant (Taurog and Chaikoff, 1946c). Thus the mean value for this percentage was 31, with a standard error of 0. 5. Furthermore, as profound a change in the condition of the thyroid as that produced by hypophysectomy failed to alter the percentage of the iodine present in the gland in the form of thyroxine (Taurog, Chaikoff and Bennett, 1946). These findings in the rat led us to examine the ratio of thyroxine to total iodine in the thyroids of other vertebrates.

EXPERIMENTAL

Thyroid glands were excised immediately after the animals were sacrificed, and their total and thyroxine iodine content determined by a method

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previously described (Taurog and Chaikoff, 1946b). For the larger glands, 20, cc. of 2N NaOH per gram of tissue was used for hydrolysis. When less than 50 mg. of thyroid tissue was available, one cc. of the base was used. Hydrolysis was carried out on a steam bath for 12 hours. The hydrolysates of the larger glands were diluted so that 1 cc. would contain amounts of iodine corresponding roughly to those contained in smaller glands. The butyl alcohol extraction of the hydrolysates and the subsequent iodine analyses were carried out as outlined by Taurog and Chaikoff (1946b).

RESULTS

Since no attempt was made to control the iodine intake of most of the animals recorded in Table 1, the finding of considerable variation in the iodine concentration of their thyroids is not unexpected. The lowest mean value, namely 29 mg. per cent, was observed in the shark; this is of interest since the shark lives in sea water. The high values found in the dogs reflect the high iodine content of their diets; they received 2–3 mg. of iodine per day.

TABLE 1. THE PROPORTION OF TOTAL IODINE PRESENT AS THYROXINE IN THE THYROIDS OF VARIOUS VERTEBRATES

(All animals were adult and were selected without regard to sex)

Species	No. of animals	Thyroid weight		Thyroxine iodine		Total iodine		Thyroxine as per cent of total iodine
		Mean	Range	Mean	Range	Mean	Range	
		Milligrams		Milligrams per cent		Milligrams per cent		
Shark (Squalus suckleyi)	8	84	37–160	8	4–14	29	17–49	27.9±0.5‡
Turtle (Pseudemys scripta troostii)	12	173	63–330	41	16–63	126	74–180	31.8±1.3
Chicken (White Leghorn)	15	56	17–97	27	9–51	105	35–201	25.0±0.7
Turkey	4	108	85–140*	36	25–54	143	100–200	24.7±0.7
Guinea Pig	8	57	45–72	12	6–24	42	25–89	28.3±1.2
Rabbit	12	133	90–220	15	4–34	50	17–98	28.9±1.1
Dog	8	833	461–1170	74	56–98	230	160–300	32.0±0.6
Rat	15	26	18–30	12	6–18	41	19–58	29.3±0.6
Horse	6	11,200	9800–12,100†	23	15–31	75	59–102	29.6±1.2
Cattle	6	*		41	19–59	128	67–199	31.6±1.0
Sheep	12	977	675–1260†	40	13–63	133	44–222	29.7±0.7

* Incomplete glands were obtained.
† Weights of single lobes.
‡ Standard error of the mean

$$\sigma_{\bar{x}} = \sqrt{\frac{\sum_i (x_i - \bar{x})^2}{n(n-1)}}$$

Despite the fact that among the 11 species studied the iodine concentrations in individual thyroids fluctuated from 17 to 300 milligrams per cent,—an eighteenfold variation,—the fraction of total iodine found in the form of thyroxine remained relatively constant. The values for thyroxine iodine expressed as a percentage of total iodine were found to be within the range of 25 to 32.*

* In comparing the variation of the ratios between and within species, the variance ratio, F, was found to be 6.45 (Snedecor, 1938). Although this value indicates that a statistically significant difference exists among some of the means of the ratios shown in the last column of Table 1, this difference is obviously quite small.

DISCUSSION

Several investigators have compared the thyroxine and total iodine levels in the thyroid glands of various animals. Baumann *et al.* (1942) found (by Blau's method) that about one third (28–36 per cent) of the iodine of the thyroid of the turtle, the alligator, and the dog appeared in the thyroxine fraction. With the same method, Michel and Lafon obtained similar values for pork and beef thyroids. The results obtained in both of these investigations are in close agreement with the findings presented here. According to Dey *et al.* (1945), the ratio of thyroxine to total iodine is the same in shark and cattle thyroids; but since Harington and Randall's method was used, their ratios are higher than ours. Parkes (1946) has also emphasized the close relation between acid-insoluble iodine (thyroxine) and total iodine in dried preparations of various mammalian thyroids.

TABLE 2. THE EFFECT OF THYROTROPIC HORMONE ON IODINE FRACTIONS OF RAT THYROIDS

(Each animal received two daily subcutaneous injection of 40 units* of thyrotropic hormone)

Treatment	No. of rats	Mean body wt.	Mean thyroid wt.	Mean thyroxine iodine	Mean total iodine	Mean ratio and S.E.†
		gm.	mg.	mg. per cent	mg. per cent	per cent
Controls	5	222	21	16	53	30.2 ± 0.7
Thyrotropic Hormone (7 days)	5	210	68	1.0	5.7	17.5 ± 0.6

* We are indebted to Dr. D. A. McGinty of Parke, Davis and Co., Detroit, for the supply of thyrotropic hormone. A unit is defined as the total dose in mg. which when injected subcutaneously over a period of 4 days into 180–200 gm. guinea pigs produces on the fifth day minimal but definite hyperplasia of the thyroid in all of 6 animals.

† S.E. = Standard Error of the Mean.

The ratio of thyroxine iodine to total iodine thus remains relatively constant from species to species and from animal to animal within in a given species. Although no explanation can be offered at present for this constancy, it is difficult to visualize how it could be so widespread unless the mechanism involved in the synthesis of thyroxine were quite similar throughout the vertebrates.

The normal relation between thyroxine and diiodotyrosine is upset during the prolonged injection of thyrotropic hormone. In rats that were injected daily for 7 days with thyrotropic hormone, the ratio of thyroxine iodine to total iodine was reduced to 17.5 ± 0.6 (Table 2). This finding in the rat is in agreement with previous observations of Gutman *et al.* (1932) on thyroid tissue excised from patients suffering from hyperthyroidism. These workers regard this lowered ratio as evidence for a change in the composition of the thyroglobin formed by the hyperactive thyroid.

SUMMARY

The fraction of total iodine present as thyroxine in the thyroid gland was found to be constant in 11 vertebrates: 1 fish, 1 reptile, 2 birds, and 7 mammals.

REFERENCES

BAUMANN, E. J., N. METZGER AND D. MARINE: *Proc. Soc. Exptl. Biol. Med.* 51: 110. 1942.
BLAU, N. F.: *J. Biol. Chem.* 102: 269. 1933.
DEY, B. B., P. S. KRISHNAN AND M. GIRIRAJ: *J. Sci. and Ind. Res.* 4: 244. 1945.
GUTMAN, A. B., E. M. BENEDICT, B. BAXTER, AND W. W. PALMER: *J. Biol. Chem.* 97: 303. 1932.
HARINGTON, C. R., AND S. S. RANDALL: *Quart. J. Pharm. and Pharmacol.* 2: 501. 1929.
HARINGTON, C. R.: The Thyroid Gland, p. 91, Oxford Press, London, 1933.
LELAND, J. P., AND G. L. FOSTER: *J. Biol. Chem.* 95: 165. 1932.
MICHEL, R., AND M. LAFON: *Compt. Rend. soc. Biol.* 140: 634. 1946.
PARKES, A. S.: *J. Endocrinol.* 4: 426. 1946.
ROTTER, G., AND E. SOOS: *Arch. f. exper. Pathol.* 173: 614. 1933.
SNEDECOR, G. W.: Statistical Methods, Collegiate Press, 1938.
TAUROG, A., AND I. L. CHAIKOFF: *J. Biol. Chem.* 163: 323. 1946a.
TAUROG, A., AND I. L. CHAIKOFF: *J. Biol. Chem.* 163: 313. 1946b.
TAUROG, A., AND I. L. CHAIKOFF: *J. Biol. Chem.* 165: 217. 1946c.
TAUROG, A., I. L. CHAIKOFF, AND L. L. BENNETT: *Endocrinology* 38: 122. 1946.

EFFECTS OF STEROIDS ON LACTATION[1]

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MOST OF the theories presented to explain the absence of lactation during the second half of pregnancy at a time when the mammary gland is fully developed attribute a predominant but indirect role to the steroid secretions of the ovaries and placenta. The ovarian role is demonstrated by the following observations: lactation occurs 1) at the end of pseudo-pregnancy in the rabbit (Smith and Smith, 1933; Donahue, 1934) 2) after ovariectomy in the pregnant goat (Drummond-Robinson and Asdell, 1926) or in rats treated with gonadotrophins (Selye et al., 1933; Anselmino, Herold et al., 1936) and 3) when corpora lutea produced by treatment with testosterone involute (Laqueur, 1943). Hammond (1917) noted that lactation occurred following termination of pregnancy in rabbits after the 12th day and concluded that milk secretion was the consequence of the involution of the corpora lutea. Evidence of an inhibitory effect of the placenta has been presented by Halban (1905), Frankl (1923), Smith and Smith (1933) and Nelson (1934).

Following these observations, many attempts have been made to reproduce experimentally, with ovarian hormones and other steroids, the conditions prevailing during pregnancy in order to inhibit lactation.

Estrogens inhibit lactation in normal animals but, depending upon the dose and the time of initiation of treatment, this inhibition is partial or complete. These results were obtained in the mouse, rat, rabbit, guinea-pig, goat and cow (reviewed by Turner, 1939; Meites and Turner, 1942). Additional evidence has been presented by Huzii (1939), Edelmann and Gaunt (1941) and Fauvet (1942) in the rat, by Menzyu (1940) in the guinea-pig, by Mixner, Meites and Turner (1944) in the goat and by Folley, Scott Watson and Bottomley (1941) in the cow. Clinical investigations on the effect of estrogens upon lactating women have given contradictory results (reviewed by Novak, 1943); however, from recent well-controlled studies by Walsh and Stromme (1944) and Fields (1945) it seems that a diminution in

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the amount of milk secreted can be obtained even during active nursing. The action of estrogens is probably mediated through the ovaries, since moderate doses of estrogens have no effect in ovariectomized animals (Anselmino and Hoffman, 1936; Folley and Kon, 1938; Edelmann and Gaunt, 1941; Fauvet, 1942).

Progesterone, even at a daily dose of 15 mg., is inactive in the rat (Folley, 1942) while ethynyl testosterone, which possesses to a lesser degree the pharmacologic properties of progesterone and testosterone, diminishes milk secretion (Folley, 1942; Cohen and Stein, 1940). In humans both compounds are inactive (Abarbanel, 1941).

Among the androgens, testosterone (Robson, 1937; Folley and Kon, 1938; Huffman, 1941; Edelmann and Gaunt, 1941) but not androsterone (Robson, 1937; Folley and Kon, 1938) inhibits lactation in normal but not in ovariectomized animals. Testosterone and methyl testosterone have been used clinically with varied success (reviewed by Novak, 1943 and Petersen, 1944).

Among the hormones of the adrenal cortex, only desoxycorticosterone has been tested; it suppresses lactation in the guinea-pig (Nelson, Gaunt and Scheizer, 1943) but not in the rat (Gaunt, Eversole and Kendall, 1942; Folley, 1942).

Combined treatment with progesterone and estrogens inhibits lactation in ovariectomized rats (Huzii, 1939; Fauvet, 1942,) indicating a possible synergism between the two hormones. Anselmino et al. (1936) had already observed that crude corpus luteum extracts prevented the initiation of lactation in ovariectomized rats previously treated with chorionic gonadotrophins; the results obtained by the latter authors seem to indicate that the activity of their extract was due to the presence of small amounts of estrogens acting synergistically with the corpus luteum hormone.

In a previous paper (Barsantini, Masson and Selye, 1946) we reported that among the various steroids administered in the form of pellets only estradiol and testosterone inhibited lactation in normal rats. Since the absorption from pellets varies with the nature of the steroids (Forbes, 1942), the possibility remained that the inactivity of some compounds was due to this factor. In the present experiments we therefore decided to administer the steroids at the same dose level in the form of an oil solution.

METHODS

Female albino rats from the same colony weighing 250–300 gm. fed on purina fox chow were individually caged as soon as pregnancy could be detected by palpation. Since uniformity is important in the intrepretation of the results (Folley and Kon, 1938), the litters were reduced or brought up to six rats within 24 hours of parturition. The steroid treatment was then immediately started and whenever necessary ovariectomy was also performed. The chief criterion used to estimate the efficiency of lactation was the growth curves of sucklings which were weigbed daily. Additional infor-

mation concerning the effects of the steroids was provided by the number of deaths among the young, the variation in weight of the mothers and the maternal instinct. These experiments were carried out for 16 days, approximate time at which the young begin to eat. On the 17th day the mothers were autopsied; mammary gland, adrenals, pituitary and ovaries were removed, placed in fixative fluid, then weighed on analytical balance and sectioned for histological study. Whenever the sucklings of a litter died during the course of the experiment, the mother was immediately sacrificed in order to prevent involution of the mammary gland.

All the steroids were dissolved in cotton-seed oil except the ethynyl-compounds which were in the form of suspension because of their low solubility in oil. The daily dose of the hormones, contained in 0.8 or 0.4 cc. of vehicle, was administered in 2 sub-cutaneous injections. The same amount of oil was given to control animals.

RESULTS

The results are summarized in Table I. The steroid compounds are designated by their common names and are arranged according to the complexity of their chemical structure enumerating estrane, androstane and pregnane derivatives in this order. The effect of treatment is indicated by the mean figures on the growth rates of litters on the 1st, 5th, 10th and 16th day after parturition as well as on the percentage of survivals. The growth curve and the percentage of survivals are necessary to have a true estimate of the effects of the treatment; some compounds may inhibit the growth without causing the death of the animals or vice versa. In the latter case, a possible explanation is that more milk becomes available to the surviving young.

Examination of the results indicates that the most active compounds in the non-operated lactating females are: estradiol, testosterone, androstenediol and dehydro-iso-androsterone. With a daily dose of 10 mg. the inhibitory effect on growth, already visible on the 5th day, was fairly marked on the 10th day and by the 16th day no young survived.

The dose of 10 mg. of estradiol was evidently very active, since no litter was alive on the 10th day of treatment. During that short period the mean bodyweight of the mothers fell from 195 gm. to 171 gm.. Since the inhitory effect of estradiol on lactation could be attributed to its toxicity in the mothers, we decided to repeat the experiment with smaller doses. With 1 mg. a day the mortality of the sucklings was not as great as before, but still on the 16th day the body weight of the mothers had decreased from 284 g. to 251 gm.; 10γ caused the same inhibition as 1 mg. but the bodyweight increased from 250 gm. to 260 gm. It is therefore possible to inject a dose of estrogens sufficient to cause an inhibition of lactation without interference with the maintenance of weight of the lactating mothers. None of the steroids, except estradiol at the high dose level, seemed to prevent suckling or covering of the litters. Although the young were almost constantly

TABLE I. AVERAGES OF THE GROWTH RATES OF LITTERS OF RATS AFTER TREATMENT OF THE MOTHERS WITH VARIOUS STEROIDS

Treatment of mother	No. of cases	Initial weight of young	5th day		10th day		16th day	
			Weight of surviving young	% surviving	Weight of surviving young	% surviving	Weight of surviving young	% surviving
α-estradiol								
10 mg.	3 n	6.1	8.3	66	—	0	—	0
1 mg.	6 n	6.9	10.0	77	11.0	55	13.0	34
1 mg.	6 c	7.1	10.0	91	15.8	91	23.3	91
10 γ	6 n	6.9	9.8	72	11.3	52	15.8	25
10 γ	7 c	7.1	11.0	100	17.6	95	25.3	91
Testosterone	3 n	6.3	8.7	95	10.8	95	—	0
10 mg.	4 c	7.0	11.0	100	17.7	100	24.0	100
Androstenedione	6 n	6.5	9.9	95	13.4	78	13.7	60
10 mg.	4 c	7.0	11.1	100	18.3	95	26.2	83
Androstenediol	6 n	5.6	7.7	87	9.0	41	—	0
10 mg.	5 c	6.3	9.5	100	15.6	97	18.3	80
Dehydro-iso-androsternone	4 n	6.0	8.5	85	12.6	54	—	0
10 mg.	4 c	7.0	9.9	100	14.9	100	20.6	92
Methyl-androstane-3(β), 17(α)-diol 10 mg.	3 n	6.3	9.3	84	14.1	76	15	75
Methyl-testosterone 10 mg.	12 n	6.9	11.1	94	15.6	80	20.4	78
Methyl-androstene-3(β), 17(α)-diol 10 mg.	7 n	6.9	11.2	98	15.3	96	15.4	49
Ethyl-testosterone 10 mg.	2 n	5.9	8.1	83	13.0	75	19.0	66
Progesterone	3 n	6.8	10.0	96	15.5	96	22.0	96
10 mg.	2 n*	6.0	8.7	100	13.3	100	19.0	100
Desoxycorticosterone (acetate) 10 mg.	6 n	7.0	11.4	100	17.1	97	25.5	87
Δ^5-Pregnenolone 10 mg.	9 n	6.2	10.0	98	17.4	98	25.3	98
Acetoxy-pregnenolone 10 mg.	9 n	7.0	11.7	100	19.3	98	28.2	98
Ethynyl-testosterone 10 mg.	3 n	5.8	9.5	100	14.3	100	21.8	53
Ethynyl-androstenediol 10 mg	3 n	7.5	10.5	100	13.0	66	15.0	60
	14 n	6.9	11.3	96	18.0	95	24.3	94
	4 c	6.3	10.2	94	17.7	90	26.1	85

n = normal rats.
c = ovariectomized rats.
* 25 mg./day.

attached to the nipples, they became progressively weaker and emaciated, their fur became dingy and they finally died. Histologic examination of the ovaries revealed the presence of large corpora lutea similar to those seen during pregnancy.

The results obtained in the non-operated lactating rats contrast with those found in ovariectomized animals. Ovariectomy alone had no significant effect on lactation; the growth curve was normal although there was somewhat more mortality in the operated group. We would like to point out that all these experiments were not conducted at the same period of the year; it was observed that in the fall the breeding of the animals was rather irregular and that a certain percentage of death among the litters occurred even in the control animals. The mean body weight of the young on the 16th day was during the fall approximately 19–21 gm. compared with 25–28 gm. during spring and summer. In the spayed groups treated with testos-

terone, androstenediol, dehydro-iso-androsterone and estradiol the growth curve and the mortality did not differ significantly from those of the untreated controls, indicating that the inhibitory effect of these steroids is mediated through the ovaries.

The other steroids which inhibit lactation to a lesser degree are, in order of decreasing activity: androstenedione, methyl androstenediol, methyl androstanediol, ethyl testosterone, ethynyl testosterone, ethynyl androstenediol and methyl testosterone. The inhibition of lactation began to appear only around the 10th day; although 50% or more of the young survived on the 16th day, their growth curve was definitely retarded. Presence of large corpora lutea was fairly difficult to ascertain except with androstenedione. Unfortunately, due to lack of material, it was impossible to repeat most of these experiments on ovariectomized animals. However with androstenedione we confirmed our previous findings that inhibition of lactation does not occur after removal of the ovaries.

It is surprising to note that in contrast with testosterone, methyl testosterone is almost inactive, although the two compounds have been found to possess in other respects the same pharmacologic properties (Clarke and Selye, 1943; Selye and Masson, 1943).

Methyl testosterone has a slight but definite inhibitory action on lactation. From the group of 12 litters, 3 did not live until the 10th day; in the other 9, the mortality was the same as in the control group which was run simultaneously (12% on the 16th day) but the growth rate was retarded (on the 10th day and the 16th day the average weight of the litters for both groups was respectively 15.2 gm. and 16.2 gm., and, 20.4 gm. and 25 gm.). Similar comparisons between androstenediol and its methyl and ethynyl derivatives, and between testosterone and its ethyl and ethynyl derivatives permit one to draw the conclusion that the addition of a methyl, ethyl or ethynyl side-chain at C^{17} decreases the ability to inhibit lactation. However, due to an insufficient number of animals in some groups, it was impossible to establish any definite relationship between the length of the side-chain or saturation and the degree of inhibition.

The inactive steroids comprise progesterone—even at a daily dose of 25 mg.—acetoxy-pregnenolone, pregnenolone and desoxycorticosterone. Comparison of the progesterone-treated group with the control groups seems to indicate an inhibitory effect of progesterone on the growth curve, although the mortality was almost nil. This experiment was conducted during the fall and the growth curve was identical to that of the controls run simultaneously.

It has been suggested from clinical observations that the inhibition of lactation could not easily be obtained when lactation was fully established. In order to verify it in the rat we decided to undertake the following experiment. Three groups of rats—2 experimental and one control group—were allowed to lactate without treatment until

the 8th day post-partum. At this time the mothers of the first 2 groups (1 normal and 1 spayed) received a daily dose of 0.5 mg. of estradiol given in two subcutaneous injections. The experimental procedure was otherwise identical to that described previously. The animals were sacrificed on the 17th day. In table II we give the mean figures on the growth rates of litters and the percentage of survival from the 8th to the 17th day after parturition. Under the influence

TABLE II. INFLUENCE OF THE TIME OF INITIATION OF TREATMENT OF MOTHERS ON THE AVERAGE GROWTH OF LITTERS

Groups and No. of cases	Treatment	8th day weight of young	13th day		17th day	
			Weight of young	% surviving	Weight of young	% surviving
I.—6R normal	estradiol 0.5 mg/day	14.0	16.6	100	15.0	30
II.—6R spayed	estradiol 0.5 mg/day	12.7	16.5	100	19.5	100
III.—6R spayed	0	12.3	17.3	100	20.2	94

of the treatment the litters continued to grow but more slowly in the normal group than in the castrate group. The difference was very marked from the 13th day when the young began to lose weight and died, while those of group II behaved like the controls of group III. It can be concluded that the inhibition of lactation can be readily obtained with estrogens independently of the time of initiation of treatment and that this inhibition is absent in spayed animals.

DISCUSSION

Meites and Turner (1942) put forward some doubts concerning the use of the growth rate of the young as a criterion of the activity of the mammary gland. Taking the estrogens as an example, they presented the following objections: 1) the estrogens excreted in the milk inhibit the growth of the young, 2) they decrease the weight of lactating mothers, 3) they make the animals nervous and prevent the letting-down of milk. These factors should certainly be taken into consideration; however they did not interfere with our experiments since the doses of steroids which suppressed lactation in normal animals were without effect in ovariectomized animals.

The inhibition of lactation with steroids depends on the presence of the ovaries since the same compounds are inactive in ovariectomized animals; furthermore the activity of the steroids seems connected with the formation of large corpora lutea. This last observation is in accordance with the old postulate that the corpus luteum prevents the onset of lactation. Recent studies by Laqueur (1943) demonstrated that initiation of lactation in testosterone-treated rats was

closely related to regressive changes in corpora lutea. The formation of corpora lutea in normal cyclic female rats has been produced by the same steroids which inhibit lactation: estrogens (Hohlweg, 1934; Wolfe, 1935; Selye et al., 1935), testosterone (Hohlweg, 1937; McKeown and Zuckerman, 1937; Wolfe and Hamilton, 1937; Laqueur and Fluhmann, 1942) and dehydro-iso-androsterone (Hohlweg, 1937). Since functionally active corpora lutea are normally present during lactation as shown by the deciduoma test, and since progesterone does not inhibit lactation, it is assumed that the corpora lutea occurring under the influence of steroids are responsible for some other secretion than the progestational hormone (Anselmino et al., 1936). If this hormone is an estrogen, the amounts secreted are fairly small, since deciduomata can be elicited in the presence of large corpora lutea produced by testosterone (Fluhmann and Laqueur, 1942). It is interesting to note that androsterone which is unable to produce luteinization of ovaries (Hohlweg, 1937) does not inhibit lactation (Folley and Kon, 1938).

Various theories have been proposed to explain the inhibition of lactation during pregnancy. According to Nelson (1936) the estrogens secreted in large amounts during pregnancy are responsible for this inhibition. They would act by suppressing the secretion of lactogenic hormone by the anterior pituitary and/or by direct inhibitory effect on the mammary gland. If that be the case, it is difficult to interpret the results obtained in ovariectomized animals. Furthermore Meites and Turner (1942a) found that estrogens as well as testosterone do not decrease but increase the lactogen content of the pituitary. According to the same authors (1942b) the secretion of prolactin which would be normally stimulated by estrogens is prevented by progesterone when the ratio of estrogens/progesterone is in the order of 1/2000. Such an hormonal condition would exist during pregnancy and therefore prevent lactation. This observation agrees with the opinion expressed in this paper that the corpora lutea formed under the influence of the steroids are responsible for the inhibition of lactation. This is also confirmed by Huzii (1939) and Fauvet (1942) who found that progesterone plus estrogens inhibit lactation in ovariectomized animals, while neither of the two hormones is active by itself. Similar experiments are already in progress in our laboratory in order to study the effect of this combined treatment in spayed lactating rats.

Since all the steroids which inhibit lactation stimulate mammary growth, Folley and Kon (1938) and Nelson, Gaunt and Schweizer (1943) advanced the idea that a substance being mammogenic possesses ipso facto the property of suppressing lactation. The fact that progesterone, which at a dose of 15 mg. is strongly mammogenic in the rat (Seyle, 1940; Selye, Borduas and Masson, 1942) failed to inhibit lactation, raises a question about the validity of this theory.

It has been recently suggested that the action of estrogens is mediated by the adrenal cortex, which enlarges and becomes hyperactive under the influence of this treatment: the resulting increased gluconeogenesis would interfere with the synthesis of the milk proteins (Mixner, Meites and Turner, 1944). This theory does not explain a normal lactation in spayed animals treated with estrogens or the inhibition of lactation with testosterone which unlike estrogens decreases the adrenal weight (Ingle, 1942).

Comparison of the present results with those reported previously (Barsantini, Masson and Selye, 1946) shows that compounds e.g. androstenediol, dehydro-iso-androsterone, etc., which were inactive when administered under the form of pellets inhibited lactation when given subcutaneously in oil solution. Since otherwise all the factors were identical, it can be assumed that the difference in the results is due to the factor dose.

SUMMARY

The effects of various steroids administered subcutaneously in oil solution have been studied both in normal and in spayed lactating rats.

In normal rats estradiol at the daily doses of 10γ and 1 mg., and, testosterone, androstenediol, dehydro-iso-androsterone at the daily dose of 10 mg. cause a marked inhibition of lactation.

Androstenedione, methyl androstenediol, methyl androstanediol, ethyl testosterone, ethynyl testosterone, ethynyl androstenediol and methyl testosterone possess an inhibitory effect which while definite is much less marked.

Progesterone, acetoxy-pregnenolone, pregnenolone and desoxycorticosterone are inactive.

The lactation inhibitory effect of all steroids so far examined is entirely mediated through the ovaries, since these steroids are inactive in spayed lactating animals. There appears to be some relationship between the luteotrophic effect of steroids and their ability to inhibit lactation.

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REFERENCES

ABARBANEL, A. R.: *Am. J. Obst. & Gynec.* 42: 110. 1941.

ANSELMINO, K. J., AND F. HOFFMANN: *Zentralbl. Gynäk.* 60: 501. 1936.

ANSELMINO, K. J., L. HEROLD, F. HOFFMANN AND R. I. PENCHARZ: *Zentralbl. Gynäk.* 60: 7. 1936.

BARSANTINI, J. C., G. MASSON AND H. SELYE: *Rev. Canad. Biol.* 5: 407. 1946.
CLARKE, E., AND H. SELYE: *J. Pharmacol. a. exper. Ther.* 78: 187. 1943.
COHEN, M. R., AND I. F. STEIN: *Am. J. Obst. & Gynec.* 40: 713. 1940.
DONAHUE, J. K.: *Physiol. Zool.* 7: 479. 1934.
DRUMMOND-ROBINSON, G., AND S. A. ASDELL: *J. Physiol.* 61: 608. 1926.
EDELMANN A., AND R. GAUNT: *Physiol. Zool.* 14: 373. 1941.
FAUVET, E.: *Arch. Gynäk.* 171: 342. 1942.*
FIELDS, F.: *Am. J. Obst. & Gynec.* 49: 385. 1945.
FLUHMANN, C. F., AND G. L. LAQUEUR: *Endocrinology* 31: 375. 1942.
FLUHMANN, C. F., AND G. L. LAQUEUR: *Proc. Soc. Exper. Biol. and Med.* 54: 223, 1943.
FOLLEY, S. J.: *Nature* 150: 266. 1942.
FOLLEY, S. J., AND S. K. KON: *Proc. Roy. Soc.*, London B 124: 476. 1938.
FOLLEY, S. J., H. M. SCOTT WATSON AND A. C. BOTTOMLEY: *J. Dairy Res.* 12: 241. 1941.
FORBES, T. R.: *Endocrinology* 30: 765. 1942.
FRANKL, O.: *Am. J. Obst. & Gynec.* 6: 399. 1923.
GAUNT, R., W. J. EVERSOLE AND E. C. KENDALL: *Endocrinology* 31: 84. 1942.
HALBAN, J.: *Arch. Gynäk.* 75: 353. 1905.
HAMMOND, J.: *Proc. Roy. Soc.*, London B, 89: 534. 1917.
HOHLWEG, W.: *Klin. Wchnschr.* 13: 92. 1934.
HOHLWEG, W.: *Klin. Wchnschr.* 16: 586. 1937.
HUZII, K.: *Jap. J. Med. Sci. Trans. Gynec.* 11: 19. 1939.*
INGLE, D. J.: *Endocrinology* 31: 419. 1942.
LAQUEUR, G. L.: *Endocrinology* 32: 81. 1943.
LAQUEUR, G. L., AND C. F. FLUHMANN: *Endocrinology* 30: 93. 1942.
MCKEOWN, T., AND S. ZUCKERMAN: *Proc. Roy. Soc.*, London B. 124: 362. 1937.
MEITES, J., AND C. W. TURNER: *Endocrinology* 30: 711. 1942a.
MEITES, J., AND C. W. TURNER: *Endocrinology* 30: 719. 1942b.
MENZYU, K.: *Jap. J. Obst. and Gynec.* 23: 25. 1940.*
MIXNER, J. P., J. MEITES AND C. W. TURNER: *J. Dairy Sci.* 27: 957. 1944.
NELSON, W. O.: *Endocrinology* 18: 33, 1934.
NELSON, W. O.: *Physiol. Rev.* 16: 488. 1936.
NELSON, W. O., R. GAUNT AND M. SCHWEIZER: *Endocrinology* 33: 325. 1943.
NOVAK, E.: *J. Clin. Endocrinology* 3: 274. 1943.
PETERSEN, W. E.: *Physiol. Rev.* 24: 340. 1944.
ROBSON, J. M.: *Proc. Soc. Exper. Biol. and Med.* 36: 153. 1937.
SELYE, H.: *Proc. Soc. Exper. Biol. and Med.* 43: 343. 1940.
SELYE, H., A. BORDUAS AND G. MASSON: *Anat. Rec.* 82: 199. 1942.
SELYE, H., J. B. COLLIP AND D. L. THOMSON: *Proc. Soc. Exper. Biol. and Med.* 30: 588. 1933.
SELYE, H., J. B. COLLIP AND D. L. THOMSON: *Proc. Soc. Exper. Biol. and Med.* 32: 1377. 1935.
SELYE, H., AND G. MASSON: *J. Pharmacol. a. exper. Therap.* 77: 301. 1943.
SMITH, G., VAN AND O. W. SMITH: *Am. J. Physiol.* 103: 356. 1933.
TURNER, C. W.: Sex and Internal Secretions, Chap. XI, 2nd Edition, Williams and Wilkins, Baltimore, 1939.
WALSH, J. W., AND W. B. STROMME: *Am. J. Obst. & Gynec.* 47: 655. 1944.
WOLFE, J. M.: *Proc. Soc. Exper. Biol. and Med.* 32: 757. 1935.
WOLFE, J. M., AND J. B. HAMILTON: *Proc. Soc. Exper. Biol. and Med.* 37: 189. 1937.

* Only abstracts were accessible to us.

INFLUENCE OF REPRODUCTIVE STAGE UPON THE FERTILITY OF GONADOTROPHIN-TREATED FEMALE RABBITS

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GONADOTROPHIC preparations have been used experimentally to bring about the ovulation of fertilizable ova in mice (Engle, 1927) rats (Cole, 1937; Evans and Simpson, 1940) and rabbits (Pincus, 1940). No adequate estimates have been made, however, of the initial fertility of the eggs in these species.

In some larger animals, there is the suggestion that the fertility of the eggs is affected by the stage of the reproductive cycle at the time of treatment. Cows treated in the early part of the estrous cycle yielded no fertilized eggs (Zavadovskii et al. 1935; Casida et al. 1940; Casida et al. 1943). but good fertility was obtained by the latter workers in those treated late in the cycle. Fertility has also been obtained in sheep treated in the follicular phase of the estrous cycle (Loginova and Lopyrin, 1938; Parkes and Hammond, 1940; Hammond et al. 1942). Murphree, et al., (1944) treated ewes in both the follicular and luteal phases of the cycle; good fertility resulted in the former phase; no fertilization of the eggs was obtained in the luteal phase. Koger (1945), however, did not note an effect of the stage of the cycle upon the fertility of treated ewes. Studies by various workers have also been made on the anestrous ewe and fertile matings have been reported. In the present study on rabbits, an investigation has been made of the influence upon egg fertilizability of the phase of the sexual cycle at the time of treatment with gonadotrophins.

EXPERIMENTAL

Both juvenile and adult rabbits from medium-weight breeds were used in this study. The juvenile females were reared in the laboratory colony and ranged from 80 to 90 days of age at the time treatment was started. The adult females were classified as (1) estrous, (2) pseudopregnant, and (3) anestrous. Only females which showed a willingness

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to accept the male the day treatment was begun were included in the estrous group. The females classified as pseudopregnant had been mated to a vasectomized male of proven sterility five days before treatment began, and therefore had active corpora lutea in the ovaries at the time treatment was initiated. The anestrous group was made up of unbred females which had not been in heat for 30 days or more previous to treatment. That these females had not ovulated recently was shown by the fact that old corpora lutea were not apparent in the ovaries at examination after treatment.

Follicle-stimulating extract of sheep pituitary glands was injected subcutaneously once each day over a period of five days. An unfractionated extract was injected intravenously on the sixth day. The follicle stimulating extract was prepared by the method of McShan and Meyer (1940); the unfractionated extract was the "B" fraction of McShan and Meyer (1943). Dosages of 0.5 gram-equivalents of the different batches of follicle stimulating extract were tested on 21-day old Sprague-Dawley rats. Nine semi-daily injections were made. On groups of three rats the ovaries averaged in weight from 22 to 105 mg. and were virtually free from luteinization. The unfractionated extracts when tested similarly gave ovaries containing corpora lutea and averaging 35 to 97 mg. from dosages of 100 milligram-equivalents.

The dosages of follicle-stimulating extract for the rabbits ranged from 2.5 to 10 gram-equivalents; some effort was made to adjust the dosage to the responses given by the rat tests. The unfractionated extract was always administered in dosages of 1 gram-equivalent.

All females were artificially inseminated intravaginally at the time of the intravenous injection, and in most cases, also 24 hours later, with semen from bucks of proven fertility. The animals were autopsied 45 to 78 hours after the intravenous injection. The eggs were flushed from the oviducts and uteri into watch glasses with physiological saline. They were then examined under the low-power binocular microscope following which, in most cases, they were transferred to hanging drop slides and examined at magnifications of 100 and 440 times. Classification was made into three groups; namely, those eggs that were normally and equally cleaved, those that were fragmenting or unequally cleaved, and those which were one-celled.

RESULTS AND DISCUSSION

A total of 2,774 eggs from 88 females was examined (Table 1). None of the eggs recovered from the rabbits treated while pseudopregnant appeared to be fertilized. This is in striking contrast to the average fertility observed in the other three groups. From 80.0 to 81.7% of the eggs were cleaving normally in the anestrous, juvenile and estrous rabbits.

The most attractive explanation for the difference in fertility between the eggs from the pseudopregnant rabbits and those from the

estrous, juvenile and anestrous rabbits is based on the presence or absence of corpora lutea. Presumably no corpora lutea were present in the animals of the last three groups at the time that treatment was initiated. Corpora lutea approximately five days of age were present in the pseudopregnant group and these were approximately 10 days of age at the time the ovulating stimulus was administered. The mechanism by which the corpora lutea prevent fertilization is yet to be determined. Assuming that the action is endocrine, it may affect the egg while it is being matured and before ovulation, or its action may be on the environment of the egg or of the sperm, or both, after

TABLE 1. FERTILITY OF OVA FROM RABBITS TREATED IN DIFFERENT REPRODUCTIVE STAGES

Stage of reproductive cycle	Number of females*	Number of eggs				Percentage of eggs presumed fertile
		Total	Equally cleaved	Fragmenting or unequally cleaved	One-celled	
Juvenile	16	478	389	4	85	81.4
Estrous	43	1443	1179	96	168	81.7
Anestrous	18	529	423	16	90	80.0
Pseudopregnant	11	324	0	67	257	0.0

* Eight animals were excluded because no eggs were recovered. Three were juvenile, three anestrous, and two were pseudopregnant.

the time of ovulation. A few sperm were noted in the washings from the oviducts of some of the pseudopregnant rabbits and in one animal, sperm were present in the albumen surrounding the unfertilized eggs. Although this indicates that the sperm traverse the female genital tract, finding them at 48 hours gives no indication of the time required for the journey or their potential fertility upon arrival in the oviduct.

SUMMARY

The fertilizability of artificially matured eggs from 88 superovulated rabbits, treated successively with follicle-stimulating and unfractionated pituitary gonadotrophic extracts, has been studied. Marked differences in fertilization rates were observed; 80.0 to 81.7% of the eggs were fertilized in the estrous, anestrous and juvenile rabbits, whereas no fertilization was observed in the pseudopregnant rabbits. The most obvious explanation is based on the presence of corpora lutea during the treatment in the pseudopregnant animals and the absence of corpora lutea in the other three groups.

REFERENCES

CASIDA, L. E., R. K. MEYER, W. H. MCSHAN AND W. WISNICKY: *Am. J. Vet. Res.* 4: 76. 1943.

CASIDA, L. E., A. NALBANDOV, W. H. MCSHAN, R. K. MEYER AND W. WISNICKY: *Proc. Am. Soc. Anim. Prod.* p. 302. 1940.

COLE, H. H.: *Am. J. Physiol.* 119: 704. 1937.
ENGLE, E. T.: *Proc. Soc. Exper. Biol. and Med.* 25: 84. 1927.
EVANS, H. M., AND M. E. SIMPSON: *Endocrinology* 27: 305. 1940.
HAMMOND J., JR., J. HAMMOND AND A. S. PARKES: *J. Agr. Sci.* 32: 308. 1942.
KOGER, M.: *Endocrinology.* 37: 165. 1945.
LOGINOVA, N. V., AND A. I. LOPYRIN: *Probl. Zivotn.* 10: 114. 1938. Cited from *Anim. Breed. Abstr.* 8: 255.
MCSHAN, W. H., AND R. K. MEYER: *J. Biol. Chem.* 135: 473. 1940.
MCSHAN, W. H., AND R. K. MEYER: *J. Biol. Chem.* 151: 259. 1943.
MURPHREE, R. L., E. J. WARWICK, L. E. CASIDA AND W. H. MCSHAN: *J. Anim. Sci.* 3: 12. 1944.
PARKES, A. S., AND J. HAMMOND: *Vet. Rec.* 52: 540. 1940.
PINCUS, G.: *Anat. Rec.* 77: 1. 1940.
ZAVADOVSKII, M. M., I. A. ESKIN AND G. F. OVSJANNIKOV: *Trud. Dinam. Razvit.* 9: 75. 1935. Cited from *Anim. Breed. Abstr.* 4: 37.

INFLUENCE OF PROGESTERONE UPON THE FERTILITY OF GONADOTROPHIN-TREATED FEMALE RABBITS

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A RELATION between the stage of the reproductive cycle at the time of treatment with gonadotrophins and the fertility of the artificially ovulated ova has been reported in rabbits (Murphree, in press), cattle (Zavadovskii et al., 1935; Casida et al., 1940, 1943) and sheep (Murphree et al., 1944). Females treated at a time when the ovaries contain functional corpora lutea have shown little or no fertility, whereas good fertility has been shown by those females treated while in the follicular phase of the sexual cycle. Although there is evidence suggesting a relationship between the presence of corpora lutea and infertility, no adequate analysis of the endocrine basis for this phenomenon has been made.

The previous studies have usually involved treatments which produced superovulation and the preparations used for the ovulating stimulus (unfractionated or luteinizing extracts of the pituitary gland injected intravenously) undoubtedly contained rather large amounts of the hormones necessary for the production of corpora lutea. In the present study, pretreatment with a follicle-stimulating extract was eliminated to avoid superovulation and to test the hypothesis that the superovulation procedure in luteal females results in the production of defective eggs. Ovulation was induced in some of the animals by an intravenous injection of a purified follicle-stimulating extract to see if there was an improvement of fertility by elimination of an excess of luteinizer.

Previously, only one stage of corpus luteum development had been investigated (in rabbits—10 days of pseudopregnancy) and it could not be judged whether the condition of infertility was one which arose immediately after ovulation, as possibly could be expected if the action was a direct humoral one from the pituitary gland, or whether the condition was gradual in its development, as might be expected from a series of complex physiological and morphological changes in

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the genital tract brought about by the action of the ovarian hormones. In the present study, the intravenous injections of either the follicle-stimulating or the unfractionated pituitary extract were made at different stages of development of the corpora lutea in pseudopregnant animals to establish the time of appearance of the infertile condition.

In addition to studying the influence upon fertility of corpora lutea of normal ovulation (i.e., of pseudopregnancy), a series of rabbits was run to determine the influence of experimentally produced corpora lutea upon the fertility of artificially ovulated eggs. It has been observed that whereas treatment of estrous rabbits (and follicular cows and ewes) with a follicle-stimulating extract, followed by an unfractionated extract, will result in superovulation and high fertility (Warwick et al., 1943; Murphree et al., 1944; Casida et al., 1943), treatment of cows and rabbits with an unfractionated preparation alone, first subcutaneously and then intravenously, resulted in little or no fertilization (Casida et al., 1943; Murphree, unpublished). It had been thought that the basis for this difference was either (1) that the infertility was due to the direct action of the unfractionated preparation upon the follicles and the eggs maturing in them, or (2) that the animals receiving the unfractionated preparation alone were ovulating during the period of the subcutaneous injections, and the resulting corpora lutea were affecting the fertility of later-ovulated eggs. Accordingly, this phenomenon was studied further in rabbits to see if there were evidence of ovulation during the treatment with the unfractionated preparation, and if so, what was the influence of these experimentally produced corpora lutea upon the fertility of eggs ovulated in their presence.

Studies were also made to test more directly the influence of the corpus luteum hormone by the administration of progesterone to estrous females for an interval of time before experimental ovulation. Further, since it is known that the effects of progesterone may be minimized or inhibited by estrogen (Hisaw and Leonard, 1930) and since induced ovulation in the estrous female, where there is presumably a high endogenous supply of estrogen, results in high fertility a series of 10-day pseudopregnant rabbits was pretreated with estrogen before experimental ovulation to see if there were any modification of the infertile condition.

METHODS

Influence of Corpora Lutea of Pseudopregnancy

Two series of rabbits were used to study the influence upon fertility of the corpora lutea of pseudopregnancy. Treatment of one series consisted of an intravenous injection of one gram-equivalent of unfractionated pituitary extract and that of the other series a like dosage, administered similarly, of follicle-stimulating extract.

The gonadotrophic hormone preparations used throughout this study were prepared by Dr. W. H. McShan of the Department of Zoology.

The follicle-stimulating extract was made by the method of McShan and Meyer (1940), the unfractionated extract was the "B" fraction of McShan and Meyer (1943). A dosage of 0.5 gram-equivalent of the follicle-stimulating extract was tested on twenty-one-day old Sprague-Dawley rats; nine semi-daily injections were made. The ovaries averaged 73 mg. in weight and were virtually free from luteinization. The unfractionated extracts when tested similarly gave ovaries containing corpora lutea and averaging 35 to 97 mg. from dosages of 100 milligram equivalents.

There were four experimental groups in each series; namely, those treated at 3, 5, 10 and 13 days of pseudopregnancy. The stage of pseudo-pregnancy was dated from the time of mating to vasectomized males of proven sterility. The control group in each series was composed of rabbits treated while in estrus. All females were artifically inseminated intravaginally at the time of the intravenous injection with semen collected from bucks of known fertility. The animals were autopsied approximately 45 hours after the intravenous injection.

Influence of Experimentally Produced Corpora Lutea

Thirty-four estrous rabbits were used in the study of the influence of experimentally produced corpora lutea. They were divided into five treatment groups. *Group A* received as subcutaneous injections 0.5 gram-equivalent of follicle-stimulating extract, daily, for five days. This was followed by an intravenous injection of one gram-equivalent of the unfractionated extract and artificial insemination on the sixth day. *Group B* received a total of 2.5 gram equivalents of the unfractionated extract in five daily subcutaneous injections. This was again followed by an intravenous injection of one gram-equivalent of the unfractionated preparation and artificial insemination on the sixth day. Autopsy was performed approximately 45 hours after the intravenous injection. *Groups C* and *D* received only the subcutaneous injections of the follicle-stimulating and unfractionated preparations, respectively, and were autopsied the day after the last injection. *Group E* was given subcutaneous injections of the unfractionated preparation and was inseminated daily during the treatment interval. Five of these rabbits were autopsied the day after the last injection; the remaining five received on the sixth day the intravenous injection and were again inseminated; these were autopsied 45 hours later. All artificial inseminations were made with pooled semen from fertile bucks.

Influence of Progesterone Injections

Progesterone was administered subcutaneously to estrous females. One group received two rabbit units a day for five successive days; a second group received a similar daily dose for a period of 10 days. On the day of the last injection with progesterone, all females were injected intravenously with an unfractionated extract and were artifically inseminated. They were autopsied approximately 45 hours later.

Influence of Estrogen Injections

Thirty-four females were given subcutaneous injections of estrogen (thirty received diethylstilbestrol dipropionate and four received estradiol benzoate) on the seventh, eight and ninth days of pseudopregnancy. This was followed on the tenth day by an intravenous injection of an unfraction-

ated pituitary extract and artificial insemination. The animals were autopsied approximately 45 hours later.

RESULTS

Influence of Corpora Lutea of Pseudopregnancy

The estrous females which were administered the unfractionated extract yielded a fertilization rate of 93.5% (Table 1). Twenty of the twenty-five eggs recovered from does which were pseudopregnant for three days were normally cleaved. The fertility of the different females in this group varied from 0 to 100 per cent, with an average of 80.0 per cent of fertilized eggs. At the fifth day of pseudopregnancy, 11 of the 50 recovered eggs were normally cleaved. Here the range in fertility for the different females was 0 to 77%, with an average of

TABLE 1. INFLUENCE OF REPRODUCTIVE STAGE UPON FERTILITY

Stage of the reproductive cycle	Extract	No. of females	No. of ovulation points	Number of eggs				Percentage fertility
				Total	Equally cleaved	Unequally cleaved	One celled	
Estrus	Unfrac.	6	47	46	43	1	2	93.5
3 days Pseudopreg.	Unfrac.	6	55	25	20	1	4	80.0
5 days Pseudopreg.	Unfrac.	6	53	50	11	1	38	22.0
10 days Pseudopreg.	Unfrac.	13*	115	108	6	1	101	5.6
13 days Pseudopreg.	Unfrac.	5	52	42	0	0	42	0
Estrus	Fol. Stim.	3	18	19	18	0	1	94.7
3 days Pseudopreg.	Fol. Stim.	2†	10	3	0	0	3	0
5 days Pseudopreg.	Fol. Stim.	7	24	15	5	1	9	33.3
10 days Pseudopreg.	Fol. Stim.	5	36	21	0	1	20	0
13 days Pseudopreg.	Fol. Stim.	5	27	19	0	0	19	0

* Does not include 1 female with 12 ovulation points, but from whom no eggs were recovered.
† Does not include 3 females with 7, 7, 3 ovulation points, but from whom no eggs were recovered.

22%. At 10 days, one of the 13 females yielded 6 fertilized eggs, resulting in 5.6% fertilized eggs for this group. At 13 days, none of the eggs was fertilized. Hence, there is seen to be a decline in fertility from 93.5% in animals with no corpora lutea in the ovaries to 0% in those with thirteen-day old corpora.

The estrous females which were treated with the follicle-stimulating extract were highly fertile, yielding 94.7% fertilized eggs. Only three eggs were recovered from the five rabbits which had been pseudopregnant for three days, but none of them was fertilized. Those rabbits pseudopregnant for five days yielded 33% fertilized eggs, and no fertilized eggs were recovered at the ten and thirteen-day stages.

A significantly larger number of ovulation points was present in the series receiving the unfractionated preparation than in the one treated with the follicle-stimulating extract (mean difference: $3.7 \pm .35$, $P < .01$). Also, the young corpora lutea produced by the unfractionated preparation were in general better formed than those in the other series. Twenty-six per cent of the young corpora lutea in the rabbits treated with the follicle-stimulating preparation were corpora hemorrhagica distended with fluid; the comparable figure in

the animals receiving the unfractionated extract was 1.6%. Many of these corpora hemorrhagica were accounted for by recovered eggs.

No difference was found between the two treatments in the ratio of recovered eggs to observed ovulation points, although there was a highly significant difference in recovery at the different stages. This appears to be due to the very poor recovery of eggs at the three-day stage in both series.

Influence of Experimentally Produced Corpora Lutea

Groups A and B may be directly compared: 96.2% of the eggs recovered from the seven rabbits treated with follicle-stimulating extracts were fertilized, whereas only 5.4% of the eggs from the unfractionated-treated group showed evidence of fertilization (Table 2). The ovulation rate in the former group was significantly higher than that of the latter group of animals (mean difference: 15.6 ± 6.8, $P < .05$, $> .01$).

TABLE 2. FERTILITY OF ESTROUS RABBITS PRETREATED WITH FOLLICLE-STIMULATING AND UNFRACTIONATED EXTRACTS

Group*	Number of females	Total no. of eggs	Number of Embryos		No. of 1-Celled or Fragmenting eggs	Average no. eggs per female	Mean fertility (%)
			2 cells–32 cells	Blastulae			
A	7	212	204	0	8	30.3 ± 6.5	96.2 ± 1.3
B	10	147	8	0	139	14.7 ± 1.9	5.4 ± 1.9
C	5	0	0	0	0	0 ± 0	0 ± 0
D	2	11	0	0	11	5.5 ± 0.5	0 ± 0
E	10	198	12	52	134	19.8 ± 2.8	32.2 ± 3.3

* Treatments of Groups:

A. 0.5 gram-equivalent follicle-stimulating extract subcutaneoulsy for 5 days; intravenous injection of unfractionated extract and artificial insemination on 6th day; autopsied 8th day.

B. Same as treatment of "A" except unfractionated extract was substituted for follicle stimulating extract during the first 5 days.

C. 0.5 gram-equivalent follicle-stimulating extract subcutaneously for 5 days; autopsied 6th day.

D. Same as treatment of "C" except unfractionated extract substituted for follicle stimulating extract.

E. 0.5 gram-equivalent unfractionated extract subcutaneously for 5 days; inseminated daily; 5 of rabbits autopsied on 6th day; other 5 received 1 gram-equivalent unfractionated extract and were artificially inseminated again; autopsied 8th day.

The intravenous injection was omitted in the treatment of Groups C and D to ascertain whether ovulation was occurring during the series of subcutaneous injections. None of the five rabbits treated with follicle-stimulating extract had ovulated, but both of the unfractionated-treated does had.

The animals in Group E are comparable to Group B, except that they were inseminated daily. The over-all fertility for this group was

32.2%. The fertilized eggs ranged in development from a blastula of 4500μ (perhaps somewhat more than six days old, Warwick et al., 1943) to embryos of two cells (about 25 hours old, Lewis and Gregory, 1929). These animals were autopsied either five or seven days after the first subcutaneous injections. It appears, therefore, that eggs are being ovulated after the first subcutaneous injection and probably throughout the treatment interval. Unfertilized eggs were recovered ranging from several freshly-ovulated ova still entrapped in the oviducal plug to those recovered from the uteri, with very much shrunken cytoplasm and a considerable albumen layer considered to be at least several days old.

Eggs which were ovulated early in the treatment period showed a significantly greater incidence of fertilization than those which were ovulated later; 45.9% of the eggs recovered from the uteri were fertilized, but only 14.9% (nine of 2–4 cells, two morulae and two blastulae) of the oviducal eggs were fertilized (mean difference: 31.0 ± 6.1, $P < .01$).

Influence of Progesterone Injections

All of the eggs recovered from the females on the shorter interval of treatment were fertilized (Table 3). Those on the longer interval of treatment, however, yielded from 0 to 38% fertilized eggs for the different females, with an average of 5.2%. Hence, administration of progesterone effectively depressed the fertilization rate in the estrous rabbit, which as indicated in Table 1 was characterized by 93.5% fertilized eggs.

TABLE 3. INFLUENCE OF PROGESTERONE UPON THE FERTILITY OF THE ESTROUS RABBIT

No. of females	Total dosage of progesterone	No. of daily injections	No. of ovulation points	Number of eggs				Percentage fertility
				Total	Equally cleaved	Unequally cleaved	One-celled	
3	10 Rb. U.	5	30	24	24	0	0	100
14	20 Rb. U.	10	141	116	6	6	104	5.2

The uteri of all these females showed histologically a definite progestational reaction even in the animals where fertilization occurred.

A comparison of the number of ovulation points observed in these females with that in females which received only the ovulating stimulus of unfractionated extract showed no depression of the ovulation rate; in fact, the average number of ovulations was greater in the animals treated with progesterone, 10.1 as compared to 7.8 (mean difference: $2.3 \pm .49$, $P < .01$).

Influence of Estrogen Injections

Eight fertilized eggs were observed in four of the rabbits on the

experiment (Table 4). This represents 16% of the eggs and 12% of the rabbits from which eggs were recovered. No association appeared between fertility and the dosage of estrogen. One of the animals showing fertility received 75 μg. of diethylstilbestrol dipropionate, another 150 μg. and another 18 mg., whereas the fourth received 200 rat units of estradiol benzoate. The ovulation rate of these animals is significantly lower than that in animals which were pseudopregnant

TABLE 4. INFLUENCE OF ESTROGEN UPON FERTILITY OF THE EGGS FROM THE PSEUDOPREGNANT RABBITS

No. of females		Total dosage estrogen	No. of ovulations points	Number of eggs				Percentage fertility
Treated	From which eggs were recovered			Total	Equally cleaved	Unequally cleaved	One-celled	
5	3	75 μg. d. d.*	31	10	4	1	5	40.0
13	10	150 μg. d. d.	47	12	2	0	10	16.7
3	2	3 mg. d. d.	10	4	0	0	4	0
3	3	6 mg. d. d.	12	3	0	0	3	0
6	5	18 mg. d. d.	17	8	1	0	7	12.5
4	3	200 ru. e. b.*	25	12	1	0	11	8.3

* d. d. = diethylstilbestrol dipropionate.
e. b. = estradiol benzoate.

for 10 days and which had received only the ovulating stimulus of unfractionated extract (Table 1); there were 8.8 ovulation points per female in the latter group and an average of 4.2 in those treated with estrogens (mean difference: $4.6 \pm .69$, $P < .01$). Button and Miller (1936) reported that small doses of estrin injected into mature rats reduced the incidence of ovulation, as judged by the number of corpora lutea at autopsy. Further, in the present data, the higher dosages appear more effective than the lower dosages in cutting down on the number of ova which are ovulated: the means range from 6.2 ovulation points on the lowest dosage to 2.8 on the highest, approximately a 55% reduction in ovulation rate (mean difference: $3.4 \pm .82$, $P < .01$).

Egg recovery was rather low in the estrogen-treated animals. There was a real difference in the proportion of recovered eggs to observed ovulation points between the estrogen-treated and untreated groups, but there were no differences in the proportions recovered on the various dosages of estrogen.

Histologically, several of the treated animals presented a condition similar to menstruation; i.e., sloughing of the epithelium of the endometrium. In many, blood was found in the lumen of the horns and in some, there were bright-colored patches on the outer surface suggesting subperimetrial hemorrhage. Zondek (1940) reported uterine hemorrhage several days following cessation of estrogen treatment in immature rabbits: "The bleeding occurs from greatly dilated

blood vessels in the uterus. The blood may escape directly into the lumen forming clots that are visible microscopically (by transillumination), or it may seep into the submucosal tissues in which event bleeding can be established with certainty only in histological preparations. The cells of the uterine epithelium in the areas of hemorrhage are usually greatly flattened, and in small patches, may be loosened or sloughed away." This is very similar to the condition found in the females of the present series.

DISCUSSION

The results obtained in these studies are consistent in providing further evidence that the presence of a functional corpus luteum in the ovary at the time of treatment with a gonadotrophic extract is in some way associated with the infertility of the artificially ovulated eggs.

Elimination of the superovulation procedure has not modified the observed infertility. The 10-day pseudopregnant rabbits receiving just an intravenous injection of pituitary gonadotrophin still yielded few or no fertilized eggs, whereas the estrous females which were similarly treated showed high fertility. Essentially similar results were obtained whether the intravenous injection was of the follicle-stimulating or the unfractionated pituitary extract. Hence, the observed infertility does not appear to be attributable to hyperstimulation nor to excessive amounts of luteinizer.

The data furnish evidence that the condition of infertility in animals that are treated during pseudopregnancy is not one which arises at the inception of pseudopregnancy, but is built up gradually over a period of at least five days and persists until at least the 13th day of pseudopregnancy. This observation is in accord with the data presented by Wislocki and Snyder (1933) on the treatment of pregnant rabbits with pregnancy urine and anterior pituitary extracts; they did not show fertility in rabbits treated after the fifth day of pregnancy. The progressive development in the pseudopregnant rabbit of the condition of infertility following treatment suggests a gradual elaboration of an endocrine substance such as might come from a developing corpus luteum.

The result of pretreatment of estrous animals with an unfractionated preparation before administering an intravenous injection of the same preparation is further evidence suggesting that it is progestin which results in the infertility of the artificially ovulated eggs since they showed that the corpora lutea which were formed early in the treatment period and were present to exert their influence from then until termination of the experiment resulted as a general rule in greater infertility of the later ovulated eggs.

The effectiveness of progesterone in depressing fertility in the estrous rabbit further strengthens the conclusion that it is this

endocrine substance which is responsible for the infertility observed during pseudopregnancy. The finding of fertility in the presence of histological progestational proliferation in rabbits treated for only five days with progesterone suggests that there is a different threshold for proliferation than for infertility.

Although the percentage of fertilized eggs obtained in the estrogen-treated animals was low, the results seem noteworthy inasmuch as they are the only evidence of fertilization at 10 days of pseudopregnancy which was found, if one excluded as an aberrant observation the one 10-day pseudopregnant female from which fertilized eggs were recovered.

Certain incidental observations in the course of this study seem worthy of note. The follicle-stimulating extract produced significantly fewer ovulations than the unfractionated extract when the same dosages in equivalents of original powder were administered. The follicle-stimulating extract was not entirely devoid of luteinizing activity, since microscopic examination of ovaries of both normal and hypophysectomized immature rats which were used to assay the material showed that a few ovaries contained a trace of luteal tissue (McShan and Meyer, 1946). Foster and Fevold (1938) reported many instances of ovulation in the immature rabbit after intravenous administration of follicle-stimulating preparation; however FSH plus traces of LH always resulted in ovulation. Greep et al. (1942), using a swine pituitary extract, termed purified thylakentrin (FSH) obtained ovulation in the estrous rabbit. The same preparation given to hypophysectomized immature female rats resulted in growth of Graafian follicles, but no luteinization.

Although the progesterone was administered to estrous rabbits and the estrogen to pseudopregnant ones, the contrasting effects upon ovulation rates is rather striking; the number of ovulations in the progesterone-treated animals was above the controls and that in the estrogen-treated animals below the controls. A possible explanation for this phenomenon is that progesterone enhances the release of some follicle-stimulator from the rabbit pituitary and more follicles are stimulated to respond to the ovulating preparation; estrogen, by inhibiting the release of follicle-stimulator from the rabbit pituitary, would cut down on the follicles available to the ovulating stimulus.

SUMMARY

Progesterone has been shown to prevent fertilization of rabbit eggs. An association has been established between the stage of development of corpora lutea and their ability to suppress fertilization of experimentally ovulated eggs. This was true whether the corpora lutea were those of pseudopregnancy or whether they were formed artificially by administration of gonadotrophins. The corpora lutea depressed fertilization whether the numbers of eggs ovulated experi-

mentally were normal for the species or far in excess of the normal. Further, fertilization was inhibited whether the pituitary gonadotrophins used to produce ovulation were primarily follicle-stimulating in character or contained both follicle-stimulating and luteinizing substances.

Direct administration of progesterone to estrual animals for 10 days before experimental ovulation also produced a marked suppression of fertilization. Administration of estrogens, which are known to have some ability to nullify the action of progestin for 3 days previous to ovulation has resulted in a few fertilized eggs in the 10-day pseudopregnant rabbit.

The unfractionated preparation was a more efficient ovulating agent than the follicle-stimulating extract. Treatment with estrogens reduced the number of ovulation points in the 10-day pseudopregnant animals, and also lowered the proportion of recovered eggs to observed ovulation points. In some animals, the estrogens produced a condition similar to menstruation.

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REFERENCES

Button, L. L., and C. I. Miller: *Proc. Soc. Exper. Biol. & Med.* 34: 835. 1936.
Casida, L. E., R. K. Meyer, W. H. McShan and W. Wisnicky: *Am. J. Vet. Res.* 4: 76. 1943.
Casida, L. E., A. Nalbandov, W. H. McShan, R. K. Meyer and W. Wisnicky: *Proc. Am. Soc. An. Prod.* 32: 302. 1940.
Foster, M. A., and H. L. Fevold: *Am. J. Physiol.* 121: 625. 1938.
Greep, R. O., H. B. Van Dyke and B. F. Chow: *Endocrinology* 30: 635. 1942.
Hisaw, F. L., and S. L. Leonard: *Am. J. Physiol.* 92: 574. 1930.
Lewis, W. H., and P. W. Gregory: *Science* 69: 226. 1929.
McShan, W. H., and R. K. Meyer: *J. Biol. Chem.* 135: 473. 1940.
McShan, W. H., and R. K. Meyer: *J. Biol. Chem.* 151: 259. 1943.
McShan, W. H., and R. K. Meyer: *Proc. Soc. Exp. Biol. & Med.*, 61: 57. 1946.
Murphree, R. L., E. J. Warwick, L. E. Casida and W. H. McShan: *J. An. Sci.* 3: 12. 1944.
Warwick, E. J., R. L. Murphree, L. E. Casida and R. K. Meyer: *Anat. Rec.* 87: 279. 1943.
Wislocki, G. B., and F. F. Snyder: *Bull. Johns Hopk. Hosp.* 52: 379. 1933.
Zavadovskii, M. M., I. A. Eskin and G. F. Ovsjannikov: *Trud. Dinam. Razvit* 9: 75. 1935. Cited from *An. Breed. Abstr.* 4: 37. 1936.
Zondek, B.: *Proc. Soc. Exp. Biol. & Med.* 43: 570. 1940.

NOTES AND COMMENTS

THE DETERMINATION OF PROTEIN-BOUND BLOOD ESTROGEN[1]

RECENT studies on the properties of blood estrogen have suggested the existence of an equilibrium between estrogen bound to protein (estroprotein) and estrogen not so bound (Szego and Roberts, 1946; Roberts and Szego, 1946). Several procedures have previously been described which seem to determine total blood estrogen. Among these may be mentioned direct assay of whole blood or serum, dialysis in the cold (Szego and Roberts, 1946), and alcohol extraction at room temperature (Smith and Smith, 1934). On the other hand, the cold acetone precipitation method described below seems to permit separate estimation of the two main fractions.

METHOD

An outline of the extraction scheme is presented in Table I.

Approximately 100 ml. of freshly obtained blood were poured slowly into 10 volumes of cold acetone with mechanical stirring. The precipitate was allowed to settle overnight at 2°C., after which the supernatant fraction was obtained by siphoning off as much as possible, followed by centrifugation of the remainder. In order to minimize denaturation, the solvent was not permitted to evaporate completely from the protein precipitate after decantation, but was immediately replaced by cold acetone-ether (1:1), and washed rapidly at the centrifuge with 3 changes of this solvent. With each washing, the precipitate was resuspended in the solvent by means of a hollow glass rod with a large ground surface. The washings were then combined with the acetone supernatant fraction. Thus far, this procedure was an adaptation of the methods of Hardy and Gardiner (1910) and Blix (1941), which had been used for the removal of lipids from blood serum.

The acetone and ether were removed from the supernatant fraction by evaporation on the steam bath. Toward the end of this process, about 100 ml. of distilled water were added and evaporation of the organic solvents completed. The aqueous residue was then made up to 4% sulfuric acid, refluxed for 10 minutes, and, after cooling, extracted 3 times with two-thirds its volume of pure peroxide-free ether. The pooled ether extract, representing the supernatant fraction, was washed with distilled water in the separatory funnel and evaporated to a small volume. After transfer to a vial of about 15 ml. capacity and addition of a measured amount of olive oil, evaporation of the residual ether was completed. An alternative procedure, if the determination of conjugated estrogen as such was desired, was to omit refluxing and assay the supernatant fraction in saline suspension after previous evaporation of all solvents.

The protein residue was spread thinly over the inside surface of the centrifuge bottle and dried in the hood overnight. It was then ground finely in a mortar and transferred quantitatively with rinsing to a beaker contain-

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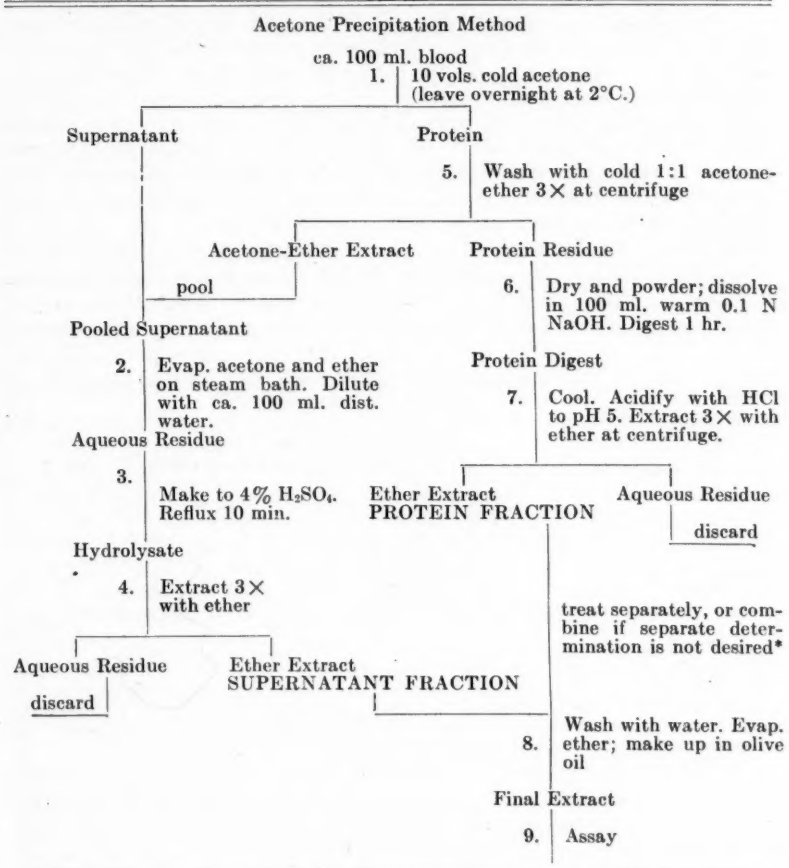
TABLE I. SIMPLIFIED SCHEME FOR THE EXTRACTION OF BLOOD ESTROGEN


Acetone Precipitation Method
ca. 100 ml. blood
1. 10 vols. cold acetone (leave overnight at 2°C.)
Supernatant
Protein
5. Wash with cold 1:1 acetone-ether 3× at centrifuge
Acetone-Ether Extract
Protein Residue
pool
6. Dry and powder; dissolve in 100 ml. warm 0.1 N NaOH. Digest 1 hr.
Pooled Supernatant
Protein Digest
2. Evap. acetone and ether on steam bath. Dilute with ca. 100 ml. dist. water.
7. Cool. Acidify with HCl to pH 5. Extract 3× with ether at centrifuge.
Aqueous Residue
3. Make to 4% H_2SO_4. Reflux 10 min.
Ether Extract
PROTEIN FRACTION
Aqueous Residue
discard
Hydrolysate
4. Extract 3× with ether
treat separately, or combine if separate determination is not desired*
Aqueous Residue
discard
Ether Extract
SUPERNATANT FRACTION
8. Wash with water. Evap. ether; make up in olive oil
Final Extract
9. Assay

* The phenolic fraction may be separated from the ether extracts at this point; this step was omitted from most of the samples.

ing about 100 ml. of warm 0.1 N. sodium hydroxide. Digestion, with intermittent stirring, was carried out for about an hour on the steam bath under a watch glass. When solution had occurred, the material was cooled and acidified to pH 5 with hydrochloric acid. It was found advantageous to add a few drops of caprylic alcohol before acidification to prevent foaming. The solution was extracted 3 times with ether in 250 ml. centrifuge bottles, using an air-driven stirrer for homogenization. After each centrifugation, the ether layer was removed with a large transfer pipette fitted with an aspirator bulb. The pooled ether extract was washed with distilled water, and the final extract was made up in olive oil or saline as described for the supernatant fraction.

COMMENT

The cold acetone extraction procedure has been applied to the determination of naturally occurring blood estrogen in several species. Typical results on the distribution of protein bound estrogen in blood have been presented in an earlier report (Szego and Roberts, 1946). Essentially equivalent total blood estrogen values were obtained on aliquots of pooled samples analyzed by this method and by the dialysis (Szego and Roberts, 1946) and alcohol precipitation (Smith and Smith, 1934) techniques. Moreover, direct assay of untreated blood or serum, when interpreted on the basis of estrogen standards in saline, gave titres which compared favorably with results on oil or saline extracts obtained by the foregoing techniques assayed on the basis of standards in the appropriate vehicle. These points are brought out in Table II, which summarizes total blood estrogen values obtained on 21 normal patients in the last trimester of pregnancy. Results are expressed

TABLE II. TOTAL ESTROGEN CONTENT OF NORMAL HUMAN PREGNANCY BLOOD DURING THE LAST TRIMESTER[1,2]

Method	Estrogenic Activity (micrograms per 100 ml.)	
	As Estriol Equivalents	As Estrone Equivalents
Acetone pptn.	1.1 (0.3 to 1.8)	46.0 (12.6 to 73.5)
Dialysis	0.8 (0.2 to 2.2)	33.5 (8.8 to 90.0)
Direct assay	1.0 (0.3 to 1.5)	41.8 (12.6 to 62.8)

[1] Assayed by a modification of the Astwood technique (1938), in which estriol in saline was found to be approximately 40 times as active as estrone in either oil or saline (Szego and Roberts, unpublished observations.)
[2] Averages and ranges in parentheses are shown.

in terms of estriol as well as estrone equivalents in view of the lack of information concerning the identity of the estrogen(s) present in human pregnancy blood.

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REFERENCES

ASTWOOD, E. B.: *Endocrinology* 23: 25. 1938.
BLIX, G.: *J. Biol. Chem.* 137: 495. 1941.
HARDY, W. B. AND S. GARDINER: *J. Physiol.* 40: p. lxviii. 1910.
ROBERTS, S. AND C. M. SZEGO: *Endocrinology* 39: 183. 1946.
SMITH, G. V. AND O. W. SMITH: *Am. J. Physiol.* 107: 128. 1934.
SZEGO, C. M. AND S. ROBERTS: *Proc. Soc. Exper. Biol. & Med.* 61: 161. 1946.
SZEGO, C. M. AND S. ROBERTS: Unpublished observations.

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THE EFFECTS OF A LARGE DOSE OF PARA-AMINO-BENZOIC ACID ON THE BODY AND ENDOCRINE GLAND WEIGHTS OF RATS: TOXIC MANIFESTATIONS

EXPERIMENTS have been conducted in this laboratory on the possible beneficial or toxic effects of large doses of individual B complex vitamins on the albino rat. Some results of parenteral administration of PABA are presented in this report.

Gordon, Goldsmith, and Charipper (1945) in an experiment fed adult male rats a diet containing 3% PABA for periods varying from 19 to 45 days. They reported no loss in body weight and a significant increase in size of the thyroid gland under such a regime. Berman (1945) injected parenterally large doses of PABA for extended periods to human patients exhibiting symptoms of thyrotoxicosis and reported improvement. In view of reports indicating that PABA may be a goiterogenic substance (MacKenzie and MacKenzie, 1943; Astwood, 1943; Gordon, Goldsmith and Charipper, 1945), the findings reported here are believed to have added significance.

METHODS

Fourteen Sherman albino male rats, thirty days of age, were selected for this experiment. The animals were litter mates from four litters. Five animals, at least one from each litter, were controls. A constant diet of dog chow checkers was fed ad libitum to all animals. Nine of the rats were injected each day intraperitoneally with 7.5 mg. of PABA. The quantity of the vitamin selected was fifteen times that which has been used in this laboratory without any apparent toxicity. Each rat was weighed every three days. At the end of forty-eight days two of the PABA-injected animals died. Then for comparison all the other animals were killed with chloroform. The adrenals, thyroid, thymus, and testes, in addition to the liver, spleen and kidneys, were removed and weighed with either an analytical or precision balance. Paired organs were weighed as one. The organ weights were expressed in per cent of body weight. They were treated statistically in order to determine the probable significance of the weight differences.

RESULTS AND DISCUSSION

The average organ weights in per cent of body weights are shown in Table 1. The adrenals of the PABA-injected animals were the only organs that showed a statistically significant increase in weight. The increase was slight. There was no significant difference in the thyroid weight,

TABLE 1. AVERAGE ORGAN WEIGHTS IN PER CENT OF BODY WEIGHT

	Adrenals	Thyroid	Thymus	Testes	Liver	Spleen	Kidneys
PABA	.0329	.0118	.114	.906	6.062	.289	1.042
Control	.0220	.0130	.210	1.068	5.213	.462	.956

as the data indicate. Actually the average thyroid weight of the injected animals was approximately 42% less than that of the controls but when ex-

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pressed in per cent of body weight there was no significant difference. It should be emphasized that the animals used in this experiment were in an actively growing period. As mentioned before, Gordon, Goldsmith, and Charipper (1945) reported an increase in size of the thyroid when PABA was fed to adult male rats.

The PABA-injected animals grew as well as the controls the first week, but during the second week their growth curve fell below that of the controls and at the end of about thirty days the difference in growth rate of the two groups became more apparent. The growth curves are shown in Figure 1. During the second week the PABA-injected animals became less active than the controls. At the fourth week they developed pronounced bloated abdomens, exhibited diarrhea and displayed but very little activity. At the end of forty-eight days, as mentioned above, two of the animals died and the others were then sacrificed for comparison.

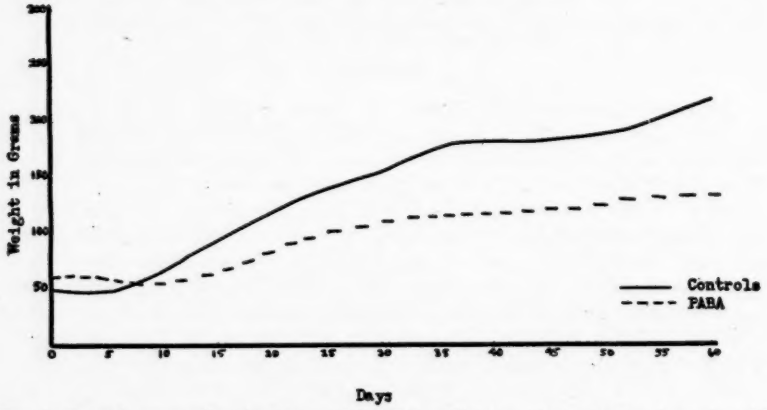

FIG. 1. Growth curve of male rats receiving 7.5 milligrams of para-amino-benzoic acid daily compared with curve of litter mate controls.

Upon autopsy of each injected animal there was found practically no storage of body fat. The small and large intestines were bloated to such an extent as to cause the liver to be pressed against the diaphragm to which it was adherent. There was no apparent obstruction in the intestines and the distention appeared to be due to collection of gas. The spleen was pressed and adhered to the abdominal wall.

The symptoms described above occurred in all the injected animals. At present the exact cause of their critical condition is not known. It may be that PABA inhibited thyroid growth to such an extent as to have been responsible for the decreased body growth. It may be possible that the symptoms were due in part or entirely to an overstimulation of an intestinal gas-forming organism or organisms by the PABA. Martin (1942) has suggested this as a possible action of PABA. It is not improbable that the injected vitamin reached the intestine, since it has been shown that PABA injected subcutaneously accumulates in the cecal contents of rats (Kornberg, Daft, and Sebrell, 1942). The distention of the intestines and the consequent dis-

placement of several of the abdominal organs, such as the liver and spleen, may have resulted in an impairment of function of these structures.

SUMMARY

PABA when injected intraperitoneally, in the quantity used in this study, into growing male rats resulted in pronounced toxic effects in all animals. Body growth was inhibited and a marked collection of gas occurred in the small and large intestines. There was a slight increase in adrenal weight with PABA administration. However, no increase in thyroid weight resulted. It is believed that more extensive study should be made of the effects of large doses of PABA on growing animals, particularly in view of its possible use as an antithyroid agent.

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REFERENCES

ASTWOOD, E. B.: *J. Pharmacol. & Exper. Therap.* 78: 79. 1943.
BERMAN, L.: *Proc. Soc. Exper. Biol. & Med.* 59: 70. 1945.
GORDON, A. S., E. D. GOLDSMITH AND H. A. CHARIPPER: *Endocrinology* 37: 223. 1945.
KORNBERG, A., F. S. DAFT, AND W. H. SEBRELL: *J. Biol. Chem.* 155: 193. 1944.
MACKENZIE, C. G., AND J. B. MACKENZIE: *Endocrinology* 32: 185. 1943.
MARTIN, G. J.: *Am. J. Physiol.* 136: 124. 1942.

THRESHOLD BLEEDING AND THE SEX SKIN IN THE CASTRATE FEMALE CHIMPANZEE[1,2]

THE SIMILARITY in chemical structure of adrenal and sex hormones and the presence of sex hormones in the urine of castrates has intrigued many with the possibility of participation by the adrenal glands in the control of cyclical genital activity. Furthermore as Parkes (1945) stated in his recent review "phenomena highly suggestive of weak estrogenic activity have been observed" in female castrates. The most striking of these has been the production of cyclical changes by constant threshold dosage with an estrogen. Bourne and Zuckerman (1941 a, b) have reported a series of experiments on the rat. In this animal the cyclical phenomenon consisted of periodic outpourings of leucocytes in an otherwise constantly cornified vaginal smear. They also reported similar morphological changes in the adrenal glands in normal and threshold cycles. They are inclined to view these histological findings as indicating a variation in rate of secretion of adrenal sex hormones and to believe that the resultant change in blood hormone level produced the altered smear picture. As an alternative, however, they do mention the possibility that the cyclical activity described might be due to a cyclic

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[1] An abstract of this paper appeared in Fed. Proc. 6: 89 (1947).

[2] The actual hormones used ethinyl estradiol (Estinyl) and anhydrohydroxy progesterone (Pranone) were generously supplied by the Schering Corporation, Bloomfield, N. J. These hormones were mixed with canned grapefruit juice and given orally.

change in threshold of the actual endorgans themselves. In the castrate female monkey Zuckerman (1941) has reported periodic menstrual flow despite unchanging dosage with an estrogen. This finding has recently been confirmed by Phelps (1946).

The concept that alterations in output of adrenal sex hormones form the basis for the so-called threshold cycles is opposed by del Castillo and di Paola (1942) who report retention of threshold cycles in the rat following adrenalectomy. Their evidence is not conclusive because they also mention the constant presence of so much accessory adrenal cortical tissue in their rats that untreated adrenalectomy is not followed by death.

The report of Clark and Birch (1946) that variations occurred in the size of the sex skin swelling of the hysterectomized and oophorectomized chimpanzee under constant dosage with follicular and luteal hormones afforded a possible method for further study of these threshold cycles.

One chimpanzee (Mimi) was available. She had been castrated several years previously and had been on intermittent sex hormone therapy since that time. The experiment is summarized in Figure 1. The curved line indicates the estimate of the size of the sex skin swelling on an arbitrary scale of

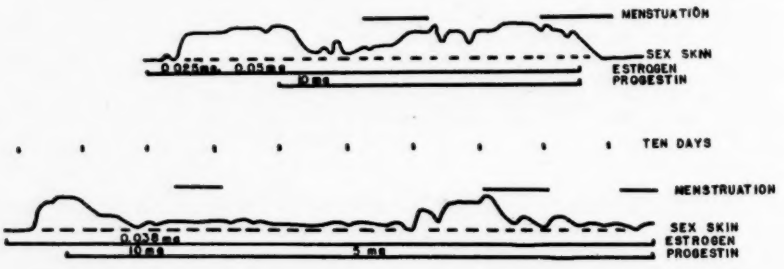

FIG. 1. Cyclic phenomena in the chimpanzee under constant dosage with sex hormones.

six units, ranging from a baseline (dashes) which represents the residual swelling seen between periods of estrogenic activity to a maximum which represents the stage in which the chimpanzee is most sexually receptive (Elder and Yerkes, 1936). Menstruation is indicated by the solid line above the sex skin swelling curve, hormone therapy below the curve and time (in ten day periods) by the dots between the two curves. It will be noted that cyclic activity was observed in two indicators. The size of the sex skin swelling waxed and waned despite constant therapy with the sex hormones and similarly periodic menstruation occurred. The time relations of these cyclical changes appear totally unrelated. Menstrual flow occurs when the sex skin swelling is increasing in size, decreasing in size or stationary. This asynchrony in the two endorgans is difficult to reconcile with the theory that such periodic changes are due to fluctuations in the output of sex hormones of adrenal origin. It is possible that one endorgan is autonomous with periodic fluctuations in threshold while the other is responding to variations in output of adrenal sex hormones but this appears, to say the least, improbable. It seems reasonable to conclude that the threshold cycles in the chimpanzee are due to periodic alterations in threshold of the responding organs. It is

probable that this same explanation can be applied to threshold cycles in other species as well.

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REFERENCES

BOURNE, G., AND S. ZUCKERMAN: *Endocrinology* 2: 268. 1941.
BOURNE, G., AND S. ZUCKERMAN: *Endocrinology* 2: 283. 1941.
DEL CASTILLO, E. G., AND G. DI PAOLA: *Endocrinology* 30: 48. 1942.
CLARK, G., AND HERBERT G. BIRCH: *Anat. Rec.* 94: 453. 1946.
PARKES, A. S.: *Physiol. Rev.* 25: 203. 1945.
PHELPS, DORIS H.: *Endocrinology* 39: 105. 1946.
ZUCKERMAN, S.: *Endocrinology* 2: 263. 1941.

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TESTIS TRANSPLANTATIONS PERFORMED BY JOHN HUNTER[1]

ALTHOUGH Berthold's fundamental experiment demonstrating the endocrine function of the avian testis (1849) is well known, it is less generally recognized that about 80 years earlier John Hunter (1728–1793) had performed successful transplantations of cock testes. Incidental reference has in more recent times been made to this pioneer experiment (Gross, 1881; Foster, 1900; Lipschütz, 1924; Pézard, Sand, and Caridroit, 1926; Anonymous, 1928; Korschelt, 1931; Olmsted, 1939, 1940), but it is worthy of special emphasis.

Unfortunately, no detailed account of the investigation now exists. Since it was Hunter's practice to record his observations with care, the assumption may be risked that his notes on the testis transplantations were among the papers destroyed after his death by his brother-in-law and executor, Sir Everard Home. From brief references in Hunter's published works, from notes taken at the lectures of John and William Hunter, and from correspondence, however, it is possible to assemble some pertinent information. John Hunter, interested in the transplantation of teeth, managed to implant dog and human teeth into roosters' combs, choosing this site because he thought the substance of the comb resembled that of the gum (Abernethy, 1817; Parkinson, 1833). He conducted a fascinating series of experiments (reviewed by Burrows, 1945) on the results of exchange of spurs between hens and roosters.

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[1] The writer is indebted to Dr. Owsei Temkin, Institute of History of Medicine, Johns Hopkins University, for criticizing the manuscript and to the staffs of the Historical Library, Yale University School of Medicine, of the Army Medical Library, and of the Sutro Branch, California State Library, San Francisco, for assistance with documents.

"I have also," says Hunter (1771), further discussing his transplantations, "frequently taken out the *Testis* of a cock and replaced it in his belly, where it has adhered, and has been nourished; nay, I have put the *Testis* of a cock into the belly of a hen with the same effect." Successful grafts adhered to the liver or the intestine. Injection of the hepatic blood vessels showed that a vascular supply to the testis had been established (J. Hunter, 1794; W. Hunter, undated MSS; Abernethy, 1817; Parkinson, 1833; Palmer, 1835; Olmsted, 1939). In an anonymous article in the *Lancet* (1928) is quoted a letter from Dr. W. Irvine, a student of Dr. William Hunter. This document, dated 17 June 1771, describes with much enthusiasm a visit to John Hunter's laboratory. According to the account, Hunter had numerous hens carrying intra-abdominal grafts of rooster testes; the latter were said to have enlarged, to contain a white liquid, and to have derived a nervous and vascular supply from their new sites of attachment. It is further recorded that, contrary to the investigator's surmise, the testes had not impregnated the hens' ova "nor altered their natural disposition."

A footnote to the *Lancet* article quotes from a stenographic record of Hunter's "Lectures on the Principles of Surgery" (Palmer, 1835; see also Parkinson, 1833, for an almost identical shorthand transcript) in which Hunter remarks to his students as he demonstrates a sample specimen that this "mode of turning hens into cocks is much such an improvement for its utility as that of Dean Swift when he proposed to obtain a breed of sheep without wool."[2] As suggested in the footnote, there is in this passage the implication that the engrafted testes had some effect on the hens. Hunter seems to have expected a sexual transformation (see quotation above) even though he poked fun at the outcome of such an experiment. (In 1780, not many years later, he published a paper on spontaneous sex reversal in the pheasant.) He had also speculated, at least, as to the effects of transplanting a rooster testis into a gander (Owen, 1861). The same source records Hunter's observation that when a cock was incompletely castrated the residual fragment underwent hypertrophy during the next eight months—possibly the earliest experimental evidence for a gonadotrophic hormone. But there seems to be no proof that Hunter was aware that male sex characters are maintained by a blood-borne secretion of the testis; this was Berthold's great contribution.

The date of the testicular transplantations can only be approximated. Hunter's book on teeth and the letter mentioned above indicate that the operations had been performed by 1771; the great British investigator was then forty-three. The writer has not found an earlier record of the work in documents left by the Hunters and their contemporaries.

SUMMARY

Evidence that avian testicular transplantations were accomplished by John Hunter in 1771 or earlier is summarized.

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[2] See *A Voyage to Laputa*, in Swift's *Gulliver's Travels*.

REFERENCES

ABERNETHY, JOHN: Physiological Lectures, Exhibiting a General View of Mr. Hunter's Physiology, and of His Researches in Comparative Anatomy. Delivered before the Royal College of Surgeons in the Year 1817. London, Longman, Hurst, Rees, Orme, and Brown. Pages 252–253.

ANONYMOUS: *Lancet* 214: 359. 1928.

BERTHOLD, A. A.: *Arch f. Anat., Physiol., u. wiss. Med.* 16: 42. 1849.

BURROWS, H.: Biological Actions of Sex Hormones. Cambridge, Cambridge University Press. 1945. Pages 106–107.

FOSTER, B.: *St. Paul Med. J.* 2: 735. 1900.

GROSS, S. D.: John Hunter and His Pupils. Philadelphia, Blakiston. 1881. Page 21.

HUNTER, J.: The Natural History of the Human Teeth: Explaining Their Structure, Use, Formation, Growth, and Diseases. London, Johnson. 1771–1778. Vol. 1, page 127.

HUNTER, J.: *Phil. Trans. Roy. Soc. London* 70: 527. 1780.

HUNTER, J.: A Treatise on the Blood, Inflammation, and Gun-Shot Wounds. London, Nicol. 1794. Pages 207, 208, 224.

HUNTER, W.: MS. Anatomical Notes taken from a Course of Dr. Hunter's Lectures by W. D. Jan. 20th 1775. Original in Sutro Branch, California State Library, San Francisco. Page 263.

HUNTER, W.: MS. Anatomical and Chirurgical Lectures Read by Dr. William Hunter and Mr. William Cruikshank (on Dissected Bodies) at their Theatre in Windmill Street, Haymarket, London. Taken in Writing by William Tempest Mercer, A Pupil attending the said Lectures, in 1775 & 1776. Original in Historical Library, Yale University School of Medicine. Page 249.

KORSCHELT, E.: Regeneration und Transplantation. II. Band: Transplantation unter Berücksichtigung der Explantation, Pflanzenpfropfung und Parabiose. Berlin, Borntraeger. 1931. Page 1095.

LIPSCHÜTZ, A.: The Internal Secretions of the Sex Glands. Cambridge, Heffer. 1924. Pages 81, 287.

OLMSTED, J. M. D.: *Bull. Histor. Med.* 7: 996. 1939.

OLMSTED, J. M. D.: *Bull. Histor. Med.* 8: 291. 1940.

OWEN, R., ed.: Essays and Observations on Natural History, Anatomy, Physiology, Psychology, and Geology. By John Hunter, F. R. S. London, Van Voorst. 1861. Vol. 1, pages 19, 36.

PALMER, J. F., ed.: The Works of John Hunter, F. R. S., with Notes. London, Longman, Rees, Orme, Brown, Green, and Longman. 1835. Vol. 1, page 391; vol. 2, page 56.

PARKINSON, J. K. W., ed.: Hunterian Reminiscences, Being the Substance of a Course of Lectures on the Principles and Practice of Surgery, Delivered by the Late Mr. John Hunter, in the Year 1785: Taken in Shorthand, and Afterwards Fairly Transcribed. London, Sherwood, Gilbert, and Piper. 1833. Page 60.

PÉZARD, SAND, AND CARIDROIT: *Arch. Biol.* 36: 541. 1926.

FURTHER OBSERVATIONS ON THE THYROXINE-LIKE ACTION OF ELEMENTAL IODINE IN THE RAT[1]

IN AN EARLIER paper (Dvoskin, 1947a), evidence was presented indicating that the subcutaneous administration of elemental iodine to the rat and chick had a thyroxine-like action. Although this action was qualitatively like that of thyroxine, it was quantitatively different, since it was only elicited with relatively high dosages of elemental iodine. Thus, while 7.5

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[1] Aided by a grant, administered by Dr. P. E. Smith, from the Rockefeller Foundation.

μg. of d,l-thyroxine[2] per day prevented the goiter due to feeding 0.1% thiouracil to the rat, 8.9 mg. of iodine per day, of which 3.3 mg. was elemental iodine and 5.6 mg. iodide, was able to exert a similar inhibitory effect. Lower dosages of iodine failed to prevent goiter formation, but exerted some inhibitory effect. Also, since iodide alone had some antigoiterogenic effect on thyroid weight, and since iodide was administered with elemental iodine in order to render the latter soluble in aqueous solution, no clear quantitative estimate could be made of the efficacy of elemental iodine alone.

It seemed of interest to use a solution containing elemental iodine alone, and to determine the minimal amount necessary to prevent the goiter due to thiouracil feeding. Further, it was hoped that the minimal effective dosage might be so small as to exert this effect without necrotizing the skin. Skin necrosis had been prominent when large dosages were administered in earlier experiments (Dvoskin, 1947a). Also, it seemed of interest to investigate further the inhibitory action of elemental iodine by determining whether the formation of intracellular colloid droplets was prevented. These droplets are known to increase in number following thiourea feeding (Thomas, 1944; Grasso, 1946), and after thyrotrophic hormone administration (Aleschin, 1939; DeRobertis, 1942; Dvoskin, 1947b), and to be absent after hypophysectomy (Dvoskin, 1947c).

METHODS

Young female rats of the Long-Evans strain were used. They ranged in weight from 57 gm. to 91 gm. at the end of the experiment. They were housed at room temperature, and fed a diet whose iodine content was unknown. The experiment was conducted during the late winter months. Thiouracil[3] was administered as 0.1% of the diet for a ten day period. One group of rats, fed the thiouracil-containing diet, received daily subcutaneous injections of 0.2 cc. of mineral oil.[4] Others, also fed the thiouracil-containing diet, were injected subcutaneously each day with 0.2 cc. of mineral oil containing either 1.0 mg. or 0.1 mg. of elemental iodine.[5,6] Untreated littermates served as controls.

At autopsy the thyroids were rapidly dissected free and weighed to the nearest milligram. They were then immediately fixed in a fluid similar to Bouin's except that it had been prepared with 70% ethanol instead of water.[7] The thyroids were then imbedded in paraffin, sectioned at 6 μ, and stained with the modified Heidenhain-Azan stain. The cell heights and intracellular colloid droplets were measured by a method outlined in earlier experiments (Dvoskin, 1947b).

[2] Twenty milligrams of 1-diiodotyrosine per day was needed to prevent the goiter due to feeding 0.1% thiouracil to rats, and this action was possibly due to thyroxine which may have contaminated this preparation. (Dempsey and Astwood, 1943).

[3] The thiouracil was supplied through the kindness of the American Cyanamid Company, Calco Chemical Division.

[4] Liquid Petrolatum, U.S.P., was used.

[5] Mineral oil was used since it was saturated and wouldn't reduce the elemental iodine. Unsaturated oils, such as sesame or peanut oil, which have a high iodine number, would not be satisfactory.

[6] The violet color of the elemental iodine in mineral oil was completely decolorized at two hours after its subcutaneous injection.

[7] This fluid preserved intracellular colloid droplets in rat thyroid glands.

RESULTS

A dosage of 1.0 mg. of elemental iodine per day completely prevented the increase in thyroid gland weight and cell height due to thiouracil feeding (Table 1). Three of the group of five animals treated with this dosage had no intracellular colloid droplets in the thyroid epithelium, while two animals had a small number of droplets.

TABLE 1. THYROID WEIGHT, CELL HEIGHT AND INTRACELLULAR COLLOID DROPLETS OF YOUNG FEMALE RATS FED 0.1% THIOURACIL FOR 10 DAYS AND INJECTED SUBCUTANEOUSLY WITH ELEMENTAL IODINE IN MINERAL OIL

Treatment	No. of animals	Thyroid weight—mg.			Cell height—microns			No. of droplets in 25 follicles		
		Av. ± σ	Ratio control	P	Av. ± σ	Ratio control	P	Av. ± σ	Ratio control	P
Untreated Controls	6	9.9±3.4	1.00	<0.01	9.1±0.8	1.00	<0.01	0.3±1.0	1	<0.01
Thiouracil Fed Controls	5	21.1±3.7	2.12		12.4±0.6	1.36		341±123	1137	
Elemental Iodine 1 mg./d	5	7.1±4.1	0.72	<0.01	8.4±0.7	0.92	<0.01	11±15	37	<0.01
Elemental Iodine 0.1 mg./d	4	16.9±2.0	1.71	>0.05	10.7±1.0	1.17	<0.01	371±78	1237	>0.6

P = *P* values are those comparing the group with thiouracil-fed controls.

A dosage of 0.1 mg. of elemental iodine per day partly inhibited the thyroid weight and cell height increase due to thiouracil feeding, but failed to prevent the formation of large numbers of intracellular colloid droplets (Table 1).

No gross necrosis of the skin was observed with these dosages of elemental iodine. Mineral oil accumulated under the skin since it remained unabsorbed.

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REFERENCES

ALESCHIN, B.: *Rev. franc. d'endocrinol.* 17: 237, 1939.
DEMPSEY, E. W., AND E. B. ASTWOOD: *Endocrinology.* 32: 509, 1943.
DE ROBERTIS, E.: *Anat. Rec.* 84: 125, 1942.
DVOSKIN, S.: *Endocrinology.* 40: 334, 1947a.
DVOSKIN, S.: *Endocrinology.* In press, 1947b.
DVOSKIN, S.: Unpublished data, 1947c.
GRASSO, R.: *Anat. Rec.* 95: 363, 1946.
THOMAS, O. L.: *Anat. Rec.* 89: 461. 1944.

[8] Ayerst, McKenna and Harrison Fellow of the American Association for the Study of Internal Secretions, 1947–1948.

ANNOUNCEMENT OF THE 1948 MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Thirtieth Annual Meeting of the Association for the Study of Internal Secretions will be held in the Palmer House, Chicago, Illinois, June 18 and 19, 1948.

The scientific sessions will be held in the Red Lacquer Room and registration will be on the fourth floor just outside the Red Lacquer Room. The Annual Dinner will be held in the same room on Friday, June 18 at 7 P.M. and will be preceded by a cocktail party, the location of which will be announced later. The Council will meet at 2 P.M. Thursday, June 17.

All members of the Association who plan to attend the Thirtieth Meeting are urged to make their reservations at once with the Palmer House, stating the time of arrival and how long they plan to remain in Chicago.